A novel soluble variant of human CEACAM1 appears due to molecular turn-over processes in contact-inhibited differentiated epithelial and endothelial cells

Dissertation

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
– Dr. rer. nat. –

vorgelegt von

Harrison Thuo Muturi

geboren in Kenia

am Fachbereich Chemie
der
Universität Duisburg-Essen

2010

Die vorliegende Arbeit wurde im Zeitraum von Mai 2007 bis November 2010 in der Arbeitsgruppe von Prof. Dr. Süleyman Ergün am Institut für Anatomie der Universität Duisburg-Essen durchgeführt.

Tag der Disputation: 02.05.2011

Gutachter:	Prof. Dr. med. Süleyman Ergün
	Prof. Dr. Matthias Epple
Vorsitzender:	Prof. Dr. Carsten Schmuck

Dedicated to my wife Emmah and my daughter Christabel

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Table of Abbreviations

A549	Lung carcinoma epithelium cells
ABC	Avidin-biotin complex
APS	Ammonium per sulphate
AS-M.5	Cutaneous angiosarcoma derived LEC
CBB	Coomassie blue brillant
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
CEACAMs	Carcinoembryonic antigen (CEA)-related cell adhesion molecules
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CSF	Cerebrospinal fluid
2-DE	2 dimensional gel electrophoresis
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylesulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleosid triphosphate
EDTA	Ethylendiamine tetraacetate
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Flourescein isothiocynanate conjugated Annexin V
GPI	glycosyl phosphatidyl inositol
h,min,sec	Hour, minute,second
HeLa-CEACAM1	Human cervix carcinoma transfected with CEACAM1
HT29	Human colon adenocarcinoma
IEF	Isoelectric focusing
Ig	Immunoglobulin
IL-2	Interleukin-2 ()
IPG	Immobilized pH gradient
ITIMs	Immunoreceptor tyrosine-based inhibitory mofits
kb	Kilobasepairs
kDa	Kilodalton
LAMP-1	Lysosomal-associated membrane protein 1
l	Liter
M	Mol/ Liter
mA	Miliamper

mg	Milligram (=10^{-3}g)
ml	Milliliter (=10^{-3}l)
µg	Microgram (=10^{-6}g)
µl	Microliter (=10^{-6}l)
mM	Millimol/ Liter
mmol	Millimol (=10^{-3}mol)
mRNA	Messenger RNA
MS	Mass spectrometry
m/z	Mass to charge ratio
ng	Nanogram'
NK	Natural killer)
OD	Optical density
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PI	Propidium Iodide and
PSG	Pregnancy-specific glycoprotein
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gelelectrophoresis
T102/3	Human colon adenocarcinoma
TEMED	Tetramethylethylendiamin
THP	Tamm-Horsfall glycoprotein
TRIS	Tris (hydroxymethyl) aminomethane
U	Units
UV	Ultra violate
V	Volt
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Vh	Volt-hour

1. Introduction

1.1 Introduction to urine

Urine is a liquid by-product of the blood that is secreted via the kidneys. It consists of 95 % water, whereas the remaining constitutes salts, amino acids and broken down blood pigments. Kidneys usually maintain plasma osmolarity by regulating water excretion. A large volume of plasma (350-400 ml/100 g of tissue per min) is filtered by the kidneys. The rate at which the plasma is filtered is known as the glomerular filtration rate, which is normally 125 ml/minute or 180 liters/day. After filtration most components in the filtrate such as water, glucose, amino acids, and salts are reabsorbed back. Then, the urine formed is passed through ureter to bladder and finally flow out through urethra (Figure 1).

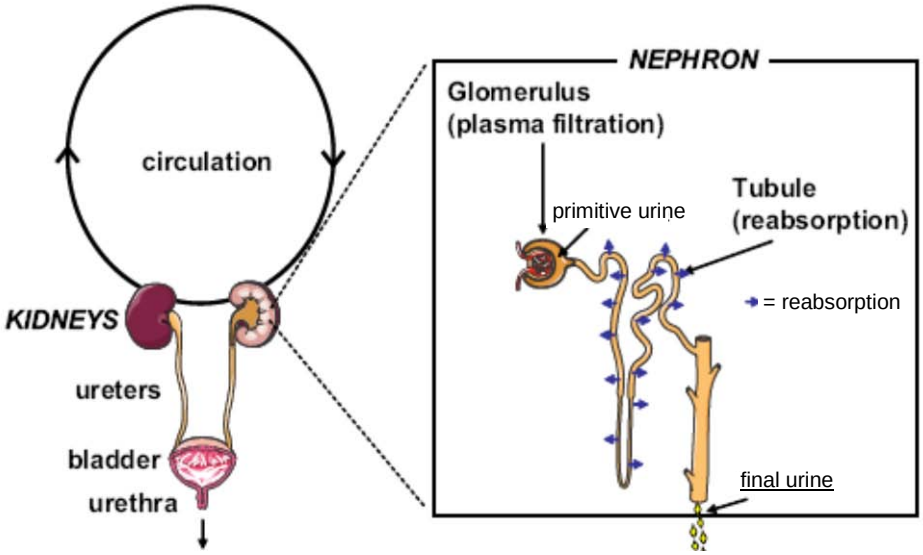


Figure 1: Urinary systems.

Plasma is filtered by the glomeruli to form “primitive urine”. However, 99% of this primitive urine is reabsorbed by the renal tubule. The remainder “final urine” exist the kidney via the ureter into the bladder. The picture obtained from (Decramer et al., 2008)

Normally, protein concentration in urine of healthy donors is very low (less than 100 mg/liter when urine output is 1.5 liters/day), whereas protein excretion is less than 150 mg/day. Excretion of more than 150 mg/day proteins are defined as proteinuria and are indicative of glomerular or reabsorption dysfunction.

1.2 Sources of urinary proteins

It is believed that proteins with a molecular weight of <15 kDa pass freely in the glomeruli whereas proteins up to 60 kDa can pass through but not freely. In addition, proteins larger than 60 kDa do not pass through the kidneys (Schenk et al., 2008). However, large

molecular weight proteins have been detected in urine. Mass spectrometry analysis revealed presence of extracellular proteins, plasma membrane proteins, and lysosomal proteins such as LAMP-1, LAMP-2 and LAMP-3 in the urine (Adachi et al., 2006). They suggested that there may be specific transport pathways for plasma membrane proteins and lysosome proteins. Additional proteomics studies have revealed many extracellular and plasma membrane protein of >60 kDa proteins in urine (Jia et al., 2009). They suggested that enrichment of these proteins in urine might be a regulatory filtration mechanism.

However, the source of proteins in urine under normal conditions were grouped into three categorize (Pisitkun et al., 2006).

1. Soluble proteins derived from largely glomerular filtration
2. Soluble proteins secreted/cleaved from the epithelial layer of glomerular membrane
3. Proteins attached with solid phase compartment from glomelular membrane

In another study of urine collected from healthy donors indicated that, 49% were soluble, 48% were contained in small cell membrane fragments, and the remaining 3% was in exosomes Table 1 (Barratt and Topham, 2007).

Protein type	% of total†	Source	Abnormalities	Notes
Soluble proteins	49	Glomerular filtration of plasma proteins (free passage of proteins < 40 kDa; normally < 150 mg/d) Some soluble proteins (e.g., Tamm-Horsfall protein) are excreted into urine by epithelial cells	Defects in glomerular filtration increase excretion of high-molecular-weight proteins (e.g., albumin) Defects in reabsorption of glomerular filtrate increase excretion of low-molecular-weight proteins	
Urinary sediment proteins‡	48	Mainly sloughed epithelial cells (from podocytes to urethral epithelia) and casts Shedding of microvilli or apoptosis of epithelial cells may generate small fragments of cell membranes	Numbers of whole cells and casts increased in many renal diseases (e.g., shedding of renal tubule cells in acute tubular necrosis and production of red cell casts in glomerulonephritis)	
Urinary exosomes§	3	All epithelia lining the urinary tract (from podocytes to urethral epithelia)	Currently unclear whether excretion of urinary exosomes is altered in diseases of the kidney and urinary tract	Many cell types secrete exosomes (B cells, T cells, platelets, enterocytes) which can be identified in plasma and may also be filtered into urine

Table 1: Sources of urinary proteins.

1.2.1 Soluble proteins derived from glomerular filtration

Most soluble proteins in urine are derived largely from glomerular filtration. The glomerular filter effectively retards passage of high molecular weight proteins. However, even with very low sieving coefficients, proteins that are abundant in the blood plasma such as albumin and various globulins can pass the glomerular filter in substantial amounts to enter the lumen of the nephron. Most of the proteins and peptides that pass the glomerular filter are scavenged and proteolyzed in the proximal tubule by highly specialized apical uptake processes that involves receptor-like recognition of the polypeptides (Christensen and Birn, 2001; Christensen, 2002). Defect in the glomerular filtration increases the excretion of high molecular weight proteins.

1.2.2 Soluble proteins cleaved from glomelular membrane

These are membrane-bound proteins that are proteolycally cleaved from the epithelial cells of urinary tract for example Tamm-Horsfall glycoprotein (THP) also known as uromodulin. It is a glycoprotein with molecular weight of approximately 85 kDa (Serafini-Cessi et al., 1993). Uromodulin is the most abundant protein in urine of healthy donors. It is usually secreted by the thick ascending limb of Henle loop, a nephron segment downstream from the proximal tubule (Serafini-Cessi et al., 2003).

1.2.3 Solid phase proteins

Proteins in urine are also found in urine sediments. Urine sediments consist mainly of whole cells, cells debris, and leukocytes. They are normally secreted into the urine from all renal tract epithelial cells. Recently tiny vesicles called “exosomes” were discovered in normal urine (Pisitkun et al., 2004a). Exosomes usually contain vesicular membranes and intracellular components of the urinary system. They may also contain proteins that are altered in abundance or physical properties in association with various renal diseases. In addition sediment proteins can be components of small fragments of cell membranes that are delivered to the urinary space from urinary tract. These may be due to budding off parts of the outer cell membrane or apoptotic cells.

1.3 Urine as a source of biomakers

A biomaker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic indication. Urine provides several advantages compared to serum or plasma and cerebrospinal fluid (CSF).

It is noninvasive

1. Easy to be obtained in large amounts.

2. Proteins and peptides in urine are quite stable.
3. The amount and composition of urinary proteome directly reflect changes in functions of the kidneys and the urogenonital tract.

On the other hand use of urine has some disadvantages as follows:

1. Urine contains low protein concentration and this varies between individual's samples.
2. In addition urine contains high levels of salts.
3. Urine may contain bacteria from lower urogenital tract. In addition, storage at room temperature for more than 6 hours or at 4°C for more than 24 hours encourages more bacteria to grow. Therefore, addition of sodium azide or boric acid to prevent bacterial contamination and grow is highly recommended.
4. Some proteins precipitate down when urine is stored at 4°C and at -20°C and they are not easily redissolved. It is recommended that urine sample should be immediately frozen at –80°C after collection to avoid loss of proteins.

The use of urinary biomarkers to diagnose disease is a long-standing practice. Ancient clinicians detected glucose in urine by tasting. In early times presence of albumin could be detected by the "foam test". Today large scale proteomics profiling of normal human urine samples has revealed the presence of at least 1000 different proteins and peptides (Castagna et al., 2005; Jurgens et al., 2005; Oh et al., 2004a; Smith et al., 2007; Sun et al., 2005; Pisitkun et al., 2004a). In this regard urine proteins offer a great potential in biomaker discovery of kidney and non-kidney diseases. For example decrease of Tamm-Horsfall protein concentration in urine has been found to be a good indicator of kidney stones (Ganter et al., 1999). In addition, urine proteins as cancer biomarker have been reported for prostate cancer (Theodorescu et al., 2008), and bladder cancer (Saito et al., 2005). Recently it was reported that CEACAM1 in urine can be used as a biomarker for bladder carcinoma (Tilki et al., 2009) indicating the presence of soluble CEACAM1 form in urine.

1.4 Introduction to CEACAM family

Carcinoembryonic antigen (CEA)-related cell adhesion molecules (CEACAMs) belong to the immunoglobulin (Ig) superfamily of cellular surface molecules. This gene family is located in the q13.1-2 region of human chromosome 19. To date, the human CEA gene family includes 29 different genes and pseudogenes (Olsen et al., 1994). These are subdivided into the CEA-related cell-adhesion molecules (CEACAMs) and the pregnancy-specific glycoprotein (PSGs) as shown below (Figure 2).

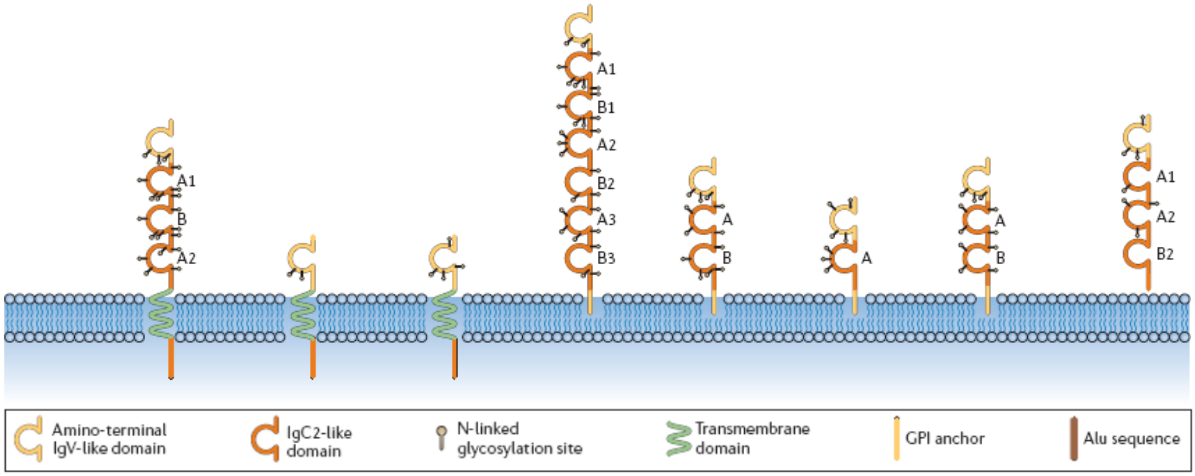


Figure 2: Schematic structure of the carcinoembryonic antigen (CEA) gene family.
The letters A and B next to the domains represent the two IgC-like domains. A modified diagram from (Gray-Owen and Blumberg, 2006)

They consist of one N-terminal variable like domain (IgV-like domain) followed by up to six domains homologous to the immunoglobulin constant region-type-2-like (IgC2-like) domains (Williams et al., 1988). The structure of the N-domain consists of a pair of β-sheets with nine β-strands (Williams et al., 1988). Another important structural feature of CEACAMs is that they are heavy glycosylated, generally more than half the receptor's molecular weight consists of carbohydrates (Williams et al., 1988). However, the level of glycosylation may vary depending upon cell type. The N-liked glycans contain high mannose and complex type oligosaccharides like lactosaminoglycans (glycoconjugates containing Galβ1,4GlcNAc mofit/mofits) type 1 and type II, terminated by fucosl- and sialyl-residues (Odin et al., 1986). CEACAM1, CEACAM3, and CEACAM4, are anchored to the membrane by short or long cytoplasmic domains, whereas CEACAM5, CEACAM6, CEACAM7, and CEACAM8 are by a glycosyl phosphatidyl inositol (GPI) anchor. CEACAM5 was the first to be discovered among all CEACAMs.

1.5 Expression of CEACAM5

CEACAM5, also known as CEA was discovered as a tumor-associated antigen in human colorectal carcinoma (GOLD and FREEDMAN, 1965). Originally, CEACAM5 was considered an oncofetal protein that is re-expressed in adult tissues only during carcinogenesis. Later, it became evident that it is expressed both in embryonic and healthy adult tissue such as colon and pancreas (Albers et al., 1988; Frangsmyr et al., 1999). Normal expression of CEACAM5 is restricted to the epithelial cells. It is expressed most abundantly on the apical surface of the gastrointestinal epithelium, but also on other mucosal epithelial cells of nasopharynx,

lung, urogenital tract and sweet glands (Thompson, 1995). Overexpression of CEACAM5 has been shown to disturb the ordered tissue architecture of several colon carcinoma cell lines (Ilantzis et al., 2002). Furthermore, CEA levels in serum are elevated during the progression of various malignant diseases such as colon, breast or lung cancers. CEA serves as a clinical tumor maker and is of important prognostic relevance in the evaluation of progressive colon tumors, an increase of it in serum indicates recurrences and residual disease(Thomson et al., 1969).

It is characterized by having seven extracellular Ig domains which includes an N-terminal domain. It has a molecular weight of approximately 180 kDa (Oikawa et al., 1987).

1.6 Expression pattern of other CEACAM family members

Similar to CEACAM5 several other members of the CEACAM family have been reported. CEACAM3 also known as CD66d has a molecular weight of approximately 35 kDa. It exists in two splice variants (CEACAM3L and CEACAM3S) with a long and a short cytoplasmic domain. It is solely expressed on human granulocytes (Nagel et al., 1993; Chen and Gotschlich, 1996).

CEACAM4 also known as CGM-7, is uniquely expressed on primary human granulocytes (Kuroki et al., 1991). However, this is not clear because it has not to shown at protein level due to lack of monospecific antibody.

CEACAM6 is alternatively known as CD66c and nonspecific cross-reacting antigen-50/90 (NCA-50/90). It was referred as NCA-50/90 because in granulocytes it detected as having 50 kDa and 90 kDa due to different glycosylation. CEACAM6 displays a fairly broad expression pattern, as it shares common sites of expression with CEACAM1. It is usually expressed in epithelia of different organs such as breast, salivary glands, pancreas, stomach, and sweat glands (Kodera et al., 1993; Metze et al., 1996; Scholzel et al., 2000b). However, no expression can be detected on the basolateral side of the gut epithelium (Hansson et al., 1989; Frangsmyr et al., 1999). It is also expressed in granulocytes, monocytes, B and T lymphocytes (Scholzel et al., 2000b; Singer et al., 2002). Significant amount of soluble CEACAM6 has been detected in serum of patients with pancreatic, breast, colon, and hepatocellular carcinomas(Kuroki et al., 1999).

CEACAM7 alternatively known as CGM2, has a molecular weight of approximately 45 kDa. It reveals an expression pattern similar to that of CEACAM5, although it is not found in granulocytes. CEACAM7 is expressed in the normal pancreatic and colon epithelium(Scholzel et al., 2000b). However, it is down regulated in colorectal tumors (Scholzel et al., 2000b).

A glycosylphosphatidyl-inositol-(GPI)-anchored glycoprotein, CEACAM8 was identified in human neutrophils 20 years ago (Buchegger et al., 1984). It is alternatively known as, CD66b, CD67 and NCA-95. Furthermore, it has a molecular weight of 90 kDa. CEACAM8 is

recognized as granulocyte-specific marker, granulocyte activation marker and as marker in patients with rheumatoid arthritis (Torsteinsdottir et al., 1999; Zhao et al., 2004). It is solely expressed in the granulocytes lineage (Berling et al., 1990). Studies have suggested that CEACAM8 is present mainly in the secondary granules, tertiary granules, secretory vesicles and on the cell surface of granulocytes (Ducker and Skubitz, 1992; Kuroki et al., 1995; Feuk-Lagerstedt et al., 1999).

CEACAM1 appears to be phylogenetically most ancient member of the CEACAM family (Stanners et al., 1992; Thompson and Zimmermann, 1988). It has been suggested, that GPI-linked CEA family members evolved from an ancestral CEACAM1-like gene by replication and mutation of a primordial transmembrane exon (Naghibalhossaini and Stanners, 2004; Stanners et al., 1992; Streydio et al., 1990). CEACAM1 was the focus of our study.

1.7 Discovery and structure of CEACAM1

Carcinoembroyonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1), also known as biliary glycoprotein 1 (BGP1) and CD66a is a member of the CEA-gene family. It has a molecular weight of approximately 120 kDa. It was first described as CEA-cross–reacting antigen from human bile (Svenberg, 1976b). Like all other expressed CEA family members, CEACAM1 consists of an N-terminal immunoglobulin variable–region like (IgV-like) domain, followed by up to three immunoglobulin constant-region type-2-like (IgC2-like) domains. The N-terminal domain consists of 108 amino acids (aa), while the IgC-like domains are found in two variants, the A and B domains. The A-domains contain 92-93 aa, whereas B-domain comprise 85-86 aa. As most Ig folds, the IgC2 set domains are stabilized by a disulfide bridge formed between paired cystine residues. The CEACAM1 gene transcript is sliced into 11 different isoforms, which differ with respects to the number of Ig-C-like domains and the length of the cytoplasmic domain (Figure 3). CEACAM1 with four extracellular immunoglobulin–like domains was named CEACAM1-4 which can either have a long or a short cytoplasmic tail. CEACAM1-4 with a long cytoplasmic tail is known as CEACAM1-4L whereas CEACAM1 with a short cytoplasmic tail is known as CEACAM1-4S. The other isoforms are CEACAM1-3L with long cytoplasmic tail and CEACAM1-3S with short cytoplasmic tail both lacking the second A-domain (Barnett et al., 1989). The other isoforms either lacks all IgC-like domains or contain some non-Ig-like regions (Barnett et al., 1993). Furthermore, they are poorly characterized in terms of function and expression pattern. It is important to note that all isoforms contain the N-domain, by which CEACAM1 mediateS homophilic binding by its non-glycosylated β-pleated sheets (Stern et al., 2005; Watt et al., 2001).

The functional differences between the isoforms containing different numbers of extracellular domains remain unclear. However, the differential splicing of exons encoding the cytoplasmic and the transmembrane region has been shown to have effects on the cellular response to CEACAM1 binding. Generally, isoforms with a long cytoplamic tail have two immunoreceptor tyrosine-based inhibitory mofits (ITIMs) which are crucial for signal transduction. In addition, the cytoplasm of isoforms with long cytoplasmic tails contains amino acids like serine, threonine and tyrosine residues that can be phosphorylation targets for signal transduction. In contrast, the isoforms of CEACAM1 with a short cytoplasmic tail lack ITIM sequences but contain sequences that can bind to globulin actin, tropomyosin, and calmodulin (Edlund et al., 1996a; Schumann et al., 2001). Furthermore, the isoforms with short cytoplamic tail are phosphorylated at the serine and threonine sites. Both isoforms are co-expressed in most CEACAM1 expressing cells and the ratio between the two isoforms seems to influence the signal outcome (Turbide et al., 1997). In addition, the ratio is not static and can vary

according to the cell type, its growth phase and activation state (Singer et al., 2000; Greicius et al., 2003).

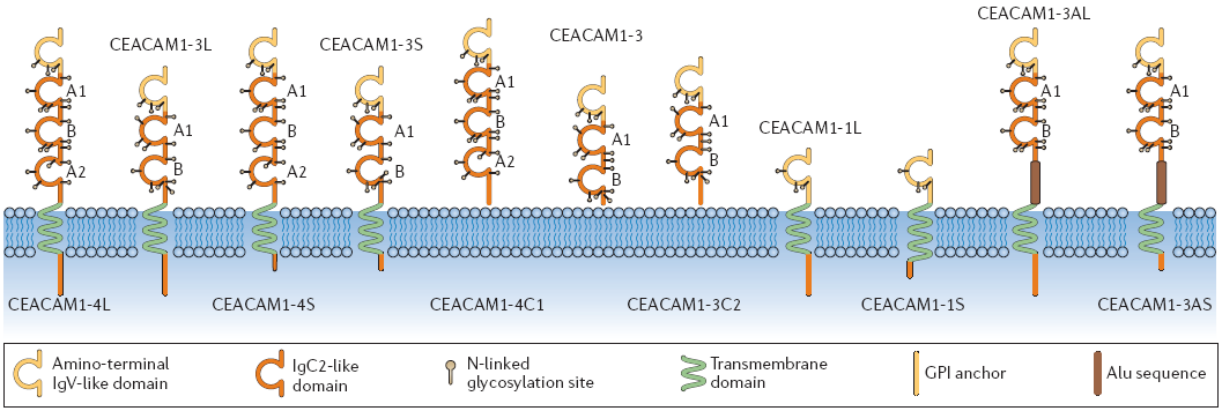


Figure 3: Naturally occurring isoforms of human CEACAM1.

The letters A and B next to the domains represent the two IgC-like domains. The number after CEACAM1 indicates the number of extracellular immunoglobulin-like domains, whereas the letter that follows this indicates the presence of either a long (L) or a short (S) cytoplasmic tail. In addition unique termini (C) or an Alu family represent a repeat sequence present within the open reading frame. Modified diagram from (Gray-Owen and Blumberg, 2006).

1.8 Expression of CEACAM1

Human CEACAM1 shows a broad cellular expression pattern. The expression differs depending on activation states and growth phase of cells. It is expressed on most epithelial cells of esophagus, gallbladder, pancreas, renal proximal tubules, stomach, colon, liver, kidney cells in the proximal tubuli, urinary bladder, prostate, cervix, squamous, endometrium and sweat glands (Prall et al., 1996; Horst and Wagener, 2004; Obrink, 1997b). Furthermore, it was detected in the extralobular and terminal ducts and myoepithelial layer of the normal mature breast (Riethdorf et al., 1997).

Furthermore, CEACAM1 is expressed on angiogenicly activated endothelial cells and is localized to the endothelial cells of new formed microvessels (Ergun et al., 2000b). Overexpression of CEACAM1 in vascular endothelial cells up-regulates potent-proangiogenic factors such as VEGF, Ang1 and Ang2 as well as lymphangiogenic factors such as VEGF-C and VEGFR-3 leading to reprogramming of vascular endothelial cells to a lymphatic phenotype (Kilic et al., 2005; Kilic et al., 2007). It is also expressed on various hematopoietic cell types such as granulocytes, B cells, T cells, natural killer (NK) cells and dendritic cells (Kammerer et al., 1998; Khan et al., 1993; Moller et al., 1996b; Singer et al., 2002; Watt et

al., 1991). CEACAM1 is rapidly upregulated on T cells by activation by cytokines such as interleukin-2 (IL-2), IL-7, IL-15 (Boulton and Gray-Owen, 2002; Kammerer et al., 1998).

1.9 Physiological functions of CEACAM1

1.9.1 Tumor suppressor

A potential tumor suppressor role of CEACAM1 was first reported from studies with hepatoma cells (Hixson et al., 1985). Later, it has been shown that mRNA levels of CEACAM1 are down-regulated in some tumors such as colorectal and prostate carcinomas (Nollau et al., 1997; Lin and Pu, 1999). In addition, down regulation of CEACAM1 in prostate cancer has been shown to be associated with loss of cell polarity. Consequently, the proliferative activity of prostate cancer cells was increased four fold and expression of CEACAM1 was lost in transition to Gleason grade 4 (Busch et al., 2002). These results collectively led to hypothesis that CEACAM1 is a tumor-suppressor.

A tumor suppressor is a protein that protects normal cells from becoming malignant. However, CEACAM1 did not fulfill this because, while down regulated in several tumor types it is also up-regulated in certain tumors. e.g. in gastric carcinomas, squamaous lung cell carcinomas (Ohwada et al., 1994) and in malignant melanomas (Thies et al., 2002). In addition it was reported that CEACAM1 does not cause but rather stabilizes and maintains contact inhibition (Singer et al., 2010).Therefore, detailed mechanisms of cell growth regulation mediated by CEACAM1 are still not understood.

1.9.2 CEACAM1 in angiogenesis

The first indication that CEACAM1 is involved in vessel development emerged from the findings that it is expressed on microvessels in the developing central nervous system of rat (Sawa et al., 1994). The first detailed analyses showing the expression of CEACAM1 in angiogenicly activated endothelial cells and newly formed small tumor vessels were published a decade ago (Ergun et al., 2000b). These authors could show that vascular endothelial growth factor (VEGF) increased CEACAM1 expression in the human primary endothelial cells (Ergun et al., 2000b). Analysis with cDNA arrays revealed that CEACAM1 is induced on microvessels of the left ventricle of chronically hypoxic rats as well as upon myocardial infarction of mice (Chen et al., 2005a). The vascular endothelial growth factor (VEGF) increased CEACAM1 expression in the endothelial cells (Ergun et al., 2000b). In addition, CEACAM1 expression was also increased in H9c2 cells (embryonic rat ventricle) after incubation with VEGF (Chen et al., 2005a). However, in CEACAM1-deficient mice a poor vascularization of implanted extracellular matrix gel that contain pro-angiogenic factors such as angiopoietin-1 and VEGF was observed (Horst et al., 2006). Therefore, CEACAM1 appears to be involved in tumor angiogenesis, as it is expressed in endothelial cells of tumor-associated small vessels but not in large and quiescent blood vessels (Kilic et al., 2005;

Muller et al., 2005; Oh et al., 2004b). Moreover, CEACAM1 overexpression in human primary endothelial cells resulted in up-regulation of pro-angiogenic factors and significantly better survival of these cells (Kilic et al., 2005).

In addition, soluble CEACAM1 exhibit pro-angiogenic effect by stimulating proliferation, chemotaxis and capillary-like tube formation of human microvascular endothelial cells *in vitro.* Furthermore, it increased the vascularization of the chorioallantoric membrane of embryos *in vivo* (Ergun et al., 2000b)

Therefore, molecular mechanisms behind CEACAM1´s angiogenic properties are currently unknown.

1.9.3 Cellular adhesion molecule

CEACAM1 can engage in homophilic (CEACAM1-CEACAM1) binding interactions. These homophilic bindings are regulated by *trans*-dimerization, the phosphorylation status of its cytoplasmic tail, its binding to calmodulin, and the activity of tissue transglutaminase (Obrink, 1997a; Hunter et al., 1998). Examples of CEACAM1 function mediated by *trans*-homophilic adhesion include the establishment of tissue organization during embryonic development, neutrophils activation, inflammatory responses, regulation of T cells, and regulation of cell proliferation (Obrink, 1997b; Hammarstrom, 1999).

At the epithelial–cell surface, CEACAM1 is found mostly as a *cis*-homodimer (Hunter et al., 1996). This dimerization leads to different CEACAM1 signaling involving SRC-family kinase and SHP phosphatases (Obrink et al., 2002). The binding of calcium ions-calmodulin complex to the cytoplasmic region of CEACAM1 causes dissociation of *cis*-dimers *in vitro* (Hunter et al., 1996). It remains unknown whether the *cis*-dimers must dissociate before intercellular binding can ensue. However, recent studies have shown that *trans*-binding increases formation of *cis*-dimers (Klaile et al., 2009a; Muller et al., 2009a). CEACAM1 isoforms undergo dimerization, presumably allowing the formation of dimers (L:L, S:S; L:S). Therefore, equilibrium between monomeric and dimeric forms affects cellular responses.

CEACAM1 anchored cell membranes also appeared as microclusters of closely packed proteins (Klaile et al., 2009a). Such clustering is expected, as predicted by (Grasberger et al., 1986) who demonstrated the likelihood of cell surface proteins forming dimers and higher oligomers.

It is well established that CEACAM1 mediates intercellular interaction via its N-terminal domain (Watt et al., 2001; Markel et al., 2004a; Stern et al., 2005; Klaile et al., 2009a). In addition, it was shown that the *trans*-homophilic CEACAM1 binding induces *cis*-dimerization by an allosteric mechanism is transmitted via the N-terminal immunoglobulin-like domain (Muller et al., 2009a). Furthermore, trans-binding between the N-terminal domains changes CEACAM1 interactions with microcluster (Klaile et al., 2009a). However, it has been

suggested that the affinity of homophilc binding seems higher when multiple IgC2 domains are present (Stern et al., 2005).
CEACAM1 is also involved in interactions with other proteins like E-selectin (Dransfield et al., 1995), and pathogenic molecules (Virji, 2000; Gray-Owen et al., 1997; Hill et al., 2001).

1.9.4 CEACAM1 as pathogen receptor

The attachment of microorganisms to host surface structures is a key step for the bacterias to colonize the mucosal surfaces (Finlay and Falkow, 1997; Abraham et al., 1998).
CEACAM-specific adhesins allows various bacterias to attach, invade and internalize into the host tissues. Adhesins are component of bacterias that facilitate adhesion to receptors on other cells. Pathogens such as *Neisseria gonorrhoeae* and *Neisseria meningitides* express opacity-associated (Opa) proteins that bind to CEACAM1 expressed on the membrane of epithelial cells (Virji et al., 1996; Virji et al., 2000; Gray-Owen et al., 1997; Boulton and Gray-Owen, 2002; Wang et al., 1998). Studies have shown Opa proteins expressed by both *Neisseria* species which bind specifically to the N-terminal domain of the CEACAM1 (Gray-Owen et al., 1997; Bos et al., 1999). Furthermore, detailed studies have shown that the β-strand in the non-glycosylated GFC face of N-terminal region is usually the binding site (Cunningham et al., 1989; Nassif et al., 1999; Popp et al., 1999). The binding of these pathogens to the cells trigger an up regulation of CEACAM1 through inflammatory cytokines such as tumor necrosis factor (Muenzner et al., 2001). By this mechanism the pathogen is able to effectively colonize the host cell.
Like gonococci the *Hemophilis influenzae* bind to CEACAM1 using adhesin P5 (Virji et al., 2000) and the binding is through the N-terminal domain (Hill et al., 2001). However, other studies show that presence of A or B domains are required (Virji et al., 2000).
Furthermore, UspA1 proteins has been identified as the *Moraxella catarrhalis* CEACAM1-binding protein (Slevogt et al., 2007; Brooks et al., 2008). The binding also occurs through the N-terminal domain (Hill and Virji, 2003).
The carbohydrates in CEACAM1 appears to be recognized by enterobacteria such as *Escherichia coli* and *salmonella* strains expressing Dr family of adhesins (Leusch et al., 1991; Sauter et al., 1993). Furthermore, detailed studies have shown these interactions are via N-terminal domain (Korotkova et al., 2006). In addition, these pathogens might exploit the immunosuppressive capacity of CEACAM1.

1.9.5 CEACAM1 as regulator of immune cell responses

1.9.5.1 CEACAM1 role in T lymphocytes

CEACAM1 is expressed on human T cells (Khan et al., 1993; Moller et al., 1996b). In addition, no other CEA family member except CEACAM1-4L and CEACAM3-3L has been characterized on T cells (Singer et al., 2002). CEACAM1 is weakly expressed on the surface

of resting T cells. However, it is significantly upregulated on T cells following activation by cytokines such as IL-2, IL-7, IL-15 (Boulton and Gray-Owen, 2002; Kammerer et al., 1998) or by stimulation through CD3/CD28 (Boulton and Gray-Owen, 2002; Moller et al., 1996b). The release of cytokines leads to priming of naive $CD4^+$ T cells with a T helper 1 effector phenotype, favoring a tumor immune response (Kammerer et al., 2001; Singer et al., 2002). In intestinal T cells activation through CEACAM1-L induces an increase of nuclear factor (NF)-kb and AP1 mediated transcription (Donda et al., 2000) and thus could facilitate crosstalk between epithelial cells and T lymphocytes in intestinal immune response.

CEACAM1 can also act a co-inhibitor of T cells receptor (TCR) responses. This happens once CEACAM1 on the surface was bound by CEACAM1 specific antibodies (Kammerer et al., 1998; Morales et al., 1999), CEACAM1-specific Opa proteins of *Neisseria gonorhoeae* (Boulton and Gray-Owen, 2002) and when bound with soluble CEACAM1-Fc (Chen et al., 2004). These ligands-CEACAM1 interactions have been shown to inhibit T cells cytokine production (IL-2), proliferation, and cytotoxic activity. Furthermore, CEACAM1 on T cells lacks the sialyl-Lewis moieties found on granulocytes (Lucka et al., 2005).

1.9.5.2 CEACAM1 role in B lymphocytes

CEACAM1 is constitutively expressed on human B cells (Khan et al., 1993; Singer et al., 2002). The cytoplasmic domain of CEACAM1-L isoforms confers on FcyR11B the capacity to inhibit BCR-induced Ca^{2+} (Chen et al., 2001) mobilization a hall mark of co-inhibitory effect. This effect requires the tyrosine residues in the membrane-proximal and the ITIM of CEACAM1. This is mediated by SHP 1 and SHP 2 (Chen et al., 2004).

In contrast to inhibitory effect, CEACAM1 is a prominent regulator of BCR-induced activation of B lymphocytes (Greicius et al., 2003). Antibodies against CEACAM1 in combination with cross-linking of the BCR stimulated cell proliferation, Ig secretion, and $ß_2$ integrin-mediated homotypic adhesion, which are crucial processes in B cell function (Greicius et al., 2003).

1.9.5.3 Neutrophilic granulocytes

Neutrophils are the leukocyte type with the broadest CEACAM expression. They co-express CEACAM1, CEACAM3, CEACAM6, and CEACAM8. During neutrophilic development, CEACAM1 is expressed from the myelocyte stage and maintained through out mature neutrophils. It is significantly expressed on resting PMNs cells. Furthermore CEACAM1 expression is rapidly upregulated upon stimulation with peptides such as formyl-methionyl-leucyl-phenylalanine (fMLP), calcium ionophores and phorbol esters (Skubitz et al., 1992; Kuroki et al., 1992; Stocks and Kerr, 1993).

Ligation of CEACAM1 with monoclonal antibody addressing N-domain stimulates β2-integrin-dependent neutral aggregation (Kuijpers et al., 1993) and adherence of neutrophils to HUVECs (human umbilical vein endothelial cells) (Kuijpers et al., 1992). Furthermore,

synthetic peptides containing sequences of the N-terminal domain of CEACAM1 can initiate signal transduction in neutrophils and are involved in interaction of CEACAM1 with other ligands (Skubitz et al., 2001; Skubitz and Skubitz, 2010).

1.9.6 Role of CEACAM1 in insulin regulation

The binding of insulin to insulin-receptor (IR) activates tyrosine kinase to cause its phosphorylation and that of other endogenous substrates including CEACAM1. CEACAM1 was originally identified as a substrate of insulin receptor tyrosine kinase in rat (Rees-Jones and Taylor, 1985). In addition, the binding causes CEACAM1 to be recruited into the IR-insulin complex (Choice et al., 1998; Najjar et al., 1998). This process leads to phosphorylation of CEACAM1 at Tyr-488 which requires an intact ser-503 residue in the cytoplasmic tail (Najjar, 2002a). Furthermore, this CEACAM1 phosphorylation promotes endocytosis of the IR-insulin complex leading to insulin degradation in lysosomes (Najjar, 2002a). It has been shown that N-terminal domain is crucial for enhancing insulin endocytosis (Soni et al., 1999).

2. Materials and Methods

2.1 Materials

2.1.1 Chemical and consumables

Chemicals	Supplier
Acrylamide	Serva (Heidelberg)
Agarose	PeqLab (Erlangen)
Ampicillin	Sigma (Taufkirchen)
APS	Merck (Darmstadt)
Bovine Trypsin	Sigma-Aldrich (Steinheim)
Coomassie Brillant Blue G-250	BioRad (München)
DMEM	Gibco BRL (Karlsruhe)
DMSO	Merck (Darmstadt)
DTT	BioRad (München)
EDTA	Serva (Heidelberg)
FCS	Gibco BRL (Karlsruhe)
Hydrogen peroxide	Sigma (Taufkirchen)
Glycerol	AppliChem (Darmstadt)
Glycin	Roth (Karlsruhe)
Iodacetamide	Sigma (Taufkirchen)
LE Agarose	Biozym scientific (Oldendorf)
L-Glutamine	Gibco BRL (Karlsruhe)
Milk powder- Non fat	AppliChem (Darmstadt)
Nonidet (NP-40)	Calbiochem (Darmstadt)
Nitrocellulose-Membrane	Millipore (Germany)
Oligonucleotides (Primers)	MWG Biotech (Ebersberg)
Page Ruler Prestained Protein Ladder	Fermentas (USA)
PBS	Gibco BRL (Karlsruhe)
TEMED	Sigma (Taufkirchen)
Tricine	AppliChem (Darmstadt)
Tris	AppliChem (Darmstadt)
Triton X-100	Sigma (Taufkirchen)
Trypsin/EDTA (1x)	Gibco BRL (Karlsruhe)
Urea	Sigma (Taufkirchen)
Cell culture flasks, plates, and tubes	Falcon (Heidelberg)

Table 2: Chemicals used and their supplier

2.1.2 Kits

Product	Supplier
BCA Protein Assay Kit	Pierce (USA)
Micro BCA Protein Kit	Pierce (USA)
RNeasy Mini Kit	Qiagen (Germany)
QuantiTech Reverse Transcrition Kit	Qiagen (Germany)
Centricon Columns	Sartorius Stedim Biotech (Germany)

ReadyPrep Sequential Extraction Kit	BioRad (Germany)
IPG Strips (11.17 cm) pH 3-10	BioRad (Germany)
Criterion Precast Gel	BioRad (Germany)
DAKO QIFIKIT	DAKO (Netherlands)
TMB Liquid Substrate System	SIGMA (USA)
PNGase F Kit	NEW ENGLAND BioLabs (USA)
Ficall-paque Plus	GE Healthcare (United Kingdom)
Fluitest UST Kit	Biocon Diagnotics (Germany)
Proteoprep Blue Albumin & IgG Depletion Kit	SIGMA (USA)
PIERCE Direct IP Kit	Pierce (USA)

Table 3: Kits used and the suppliers name

2.1.3 Stock solutions and buffers

Tricine SDS polyacrylamide gels

Tricine gel buffer pH 8.45

SDS 0.3% (w/v) 1.5g

Tri-HCl (3 M) 181.71 g

Adjust to 500 ml with deionized H_2O

1x Cathode buffer pH 8.3

Tricine (0.1 M) 17.92 g

SDS 0.1% (w/v) 1 g

Tris-HCL (0.1 M) 12.1 g

Adjust to 1000 ml with deionized H_2O

1x Anode buffer pH 8.9

Tris-HCl (0.2 M) 24.23 g

Adjust to 1000 ml with deionized H_2O

SDS polyacrylamide gels

Separating gel buffer pH 8.8

SDS 0.4% (w/v) 0.8 g

Tris-HCL (1.5 M) 36.3 g

Adjust to 200 ml with deionized H_2O

Stacking gel buffer pH 6.8

SDS 0.4% (w/v) 0.4 g

Tris-HCL (0.5 M) 6.0 g

Adjust to 100 ml with deionized H_2O

10x SDS running buffer pH 8.8

Glycin (2 M) 144 g

SDS 1% (w/v) 10 g

Tris-HCL (250 mM) 30 g

Adjust to 1000 ml with deionized H_2O

1x blotting buffer pH 8.4

Glycin (133 mM) 40 g

Tris (12 mM) 6 g

Adjust to 4 liters with deionized H_2O

1X TBS Wash buffer pH 7.6

Tris-HCL (20mM) 2.42 g

NaCl (137mM) 8.01 g

Adjust to 1000 ml with deionized H_2O

Western blot detection solution A

Courmaric acid (6.8 mM) 0.112 g

Adjust to 100 ml with DMSO (H_2O free)

Blot detection solution B pH 8.5

Luminol (1.25 mM) 0.22 g

Tris-HCL (0.1M) 12.1 g

Adjust to 1000 ml with deionized H_2O

Blot detection solution C

H_2O_2 3% (v/v)	10 µl
H_2O	90 µl

Preparation of Western blot detection solution

Solution A	15 µl
Solution B	1 ml
Solution C	3 µl

5x SDS sample buffer pH 6.8 for 10 ml

Tris-HCL (500 mM)	0.61 g
Glycerol 75% (v/v)	7.5 ml
SDS 15% (w/v)	1.5 g
Bromophenole blue 0.125 % (w/v)	12.5 mg

Adjust to 10 ml with distilled H_2O

75 mM β-mercaptoethanol (fresh)

Coomassie staining solution

Acetic Acid	10% (v/v)
Ethanol	50% (v/v)
Coomassie Brillant Blue G-250	0.5% (w/v)

Commassie destaining solution

Acetic Acid	5% (v/v)
Ethanol	10% (v/v)

Silver staining solution

Fixation solution

Ethanol 50% (v/v)	500 ml
Acetic acid 12% (v/v)	120 ml
From 35% Formalin	500 µl

Adjust to 1000 ml with deionized H_2O

Wash solution

Ethanol 20% (v/v)	200 ml

Adjust to 1000 ml with deionized H_2O

Sensitizing solution

Sodium Triosulfate 0.02% (w/v)	200 mg

Adjust to 1000 ml with deionized H_2O

Staining solution (freshly prepared)

Deionized H_2O	75 ml
From $AgNO_3$ 10% (v/v)	8 ml
From 35% Formalin	76.4 µl

Adjust to 100 ml with deionized H_2O

Developing solution

Na_2CO_3 6% (w/v)	60 g
Na_2S2O_3 0.004% (w/v)	4mg
From 35% Formalin	500 µl

Adjust to 1000 ml with deionized H_2O

Terminating solution

Acetic acid 12% (v/v)	120 ml

Adjust to 1000 ml with deionized H_2O

Nitro blue staining

Reduced Glutathione buffer pH 9.6

Reduced glutathione	8 mM
Tris-HCL	0.1 M

Nitroblue tetrazolium buffer pH 9.6

NBT	1 mM
Tris	0.1 M

RIPA Lysis buffer

Phosphate buffer 0.01M pH 7.2	250 ml
KH_2PO_4	0.41 g
Na_2HPO_4	1.25 g
Phosphate buffer (Solution)	200 ml
From 10% SDS 0.1% (v/v)	2.5 ml
Triton X-100 1% (v/v)	2.5 ml
Sodium Deoxycholate 1% (w/v)	2.5 g
Sodium Flouride 50 mM	0.52 g
NaCl 150 mM	2.19 g
EDTA 2 mM	0.15 g

Adjust the volume using phosphate buffer

Adjust the 7.2 pH with 1N NaOH

Protease inhibitor cocktail (fresh)

2D-PAGE Rehydration Buffers

2D-PAGE Equilibration Buffer I

Urea	5 M
SDS	20% (w/v)
Tris-HCl (pH 8.8)	1.5 M
Glycerol	50% (w/v)
DTT (fresh)	0.5% (w/v)

2D-PAGE Equilibration Buffer II

Urea	5 M
SDS	20% (w/v)
Tris-HCl (pH 8.8)	1.5 M
Glycerol	50% (w/v)
Iodacetamide (fresh)	2.5% (w/v)

Reagent 2 (ReadyPrep Sequential Extraction Kit)

Urea	8 M
CHAPS	4% (w/v)
SB 3-10	2% (w/v)
Tris	40 mM
Biolyte3-10	0.2% (v/v)
TBP (fresh)	2 mM
75 mM β-mercaptoethanol (fresh)	
Bromphenolblue	trace

Reagent 3 (ReadyPrep Sequential Extraction Kit)

Urea	5 M
Thiourea	2 M
CHAPS	2% (w/v)
SB 3-10	2% (w/v)
Tris	40 mM
Biolyte 3-10	2% (v/v)
TBP (fresh)	2 mM
DTT (fresh)	0.5% (w/v)
Bromphenolblue	trace

50x TAE Buffer pH 7.5

Tris-Base	2 M
Glacial acetic acid	5.7% (v/v)
EDTA	50 mM

2.1.4 Equipment and appliance

Instruments	Supplier
Autoclave	Thermo Scientific (USA)
Centrifuge 5415D	Epperdorf (Humburg)
Centrifuge Sorvall Rc5c Plus	Thermo scientific (USA)
Cold Centrifuge Micro 200R	Hettich Zentrifugen (Tuttlingen)
Cold Centrifuge ROTINA 38R	Hettich Zentrifugen (Tuttlingen)
ELISA Reader	TECAN (USA)
FACS Calibur	Beck Dickinson (USA)
Gel electrophorsis Apparatus	BioRad (USA)
Gel Ducumentation system LAS-300RG	Fiji-Film (Düsseldorf)
Thermo Block	Grant (USA)
Magnetic mixers	Heidolph (Kelheim)
Voltex	IKA (Staufen)
Water Bath	WTW (Weiheim)
Cells incubator	Heraeous Instruments (Hanau)
Light Microscope	ZEISS (Jena)
PCR iCycler	BioRad (USA)
Vacuum hood	NuAire (USA)
pH Meter	WTW (Weilheim)
Rotator SB2	Stuart Stuart
Balances	Gunten (Bot-Kirchhellen)
Gel Imager uv- Systeme	Intras (Göttigen)
Shakers	IKA (Staufen)

Table 4: All equipments used

2.1.5 Antibodies

Antibody	Source	Supplier
Anti-hCEACAM1 (Clone B3)	Mouse CEACAM1	Kindly provided by Dr. Bernhard B. Singer (Institute of Anatomy, University Hospital Essen)

Anti-hCEACAM1 (Clone C5-1X)	Mouse CEACAM1	Kindly provided by Dr. Bernhard B. Singer (Institute of Anatomy, University Hospital Essen)
Anti-hCEACAM1 (Clone 18/20)	Mouse CEACAM1 CEACAM3 CEACAM5	Kindly provided by Dr. Bernhard B. Singer (Institute of Anatomy, University Hospital Essen)
Anti-hCEACAM1 (Clone 4/3/17)	Mouse CEACAM1 CEACAM5	Kindly provided by Dr. Bernhard B. Singer (Institute of Anatomy, University Hospital Essen)
Anti-hCEACAM1 (Clone 4D1/C2)	Mouse CEACAM1	Kindly provided by Professor Dr. Wagener. (Institute of Urology, University Hospital Hamburg-Eppendorf)
Anti-hCEACAM1 (Cat No. AF2244)	Mouse CEACAM1	R&D Systems
Anti-hCEACAM1 (Clone 283340)	Mouse CEACAM1	R&D Systems
Polyclonal Anti-Human Carcinoembryonic Antigen	Rabbit All CEACAM´s	DAKO (Netherlands)
Anti-hCEACAM1 (Clone 8G5)	Mouse CEACAM1	Genovac Freiburg

Table 5: primary antibodies

Antibody	Source	Working Dilution	Supplier
Anti-Mouse IgG F(ab´)2 Fragement HRP	Mouse	1:5000	Jackson ImmunoResearch
Anti-Mouse IgG (H+L) HRP	Mouse	1:5000	Jackson ImmunoResearch
Anti-Human CD25 FITC	Mouse	1:40	ImmunoTools
Anti-Human CD69 Dy647	Mouse	1:40	ImmunoTools
Anti-CD3 PE	Mouse	1:40	EXBIO Praha
Anti-Mouse IgG F(ab´)2 Fragement FITC	Mouse	1:40	Jackson ImmunoResearch

Table 6: secondary HRP antibodies

2.1.6 Cell lines and medium for cultivation

Cell line	Characteristics	Source
A549	Lung carcinoma epithelium cells	PromoChem (Wesel, Germany)
HT29	Human colon adenocarcinoma	DSMZ (Braunschaweig, Germany)
T102/3	Human colon adenocarcinoma	Kindly provided by PD Dr. Robert Kammerer (Institute of Immunology, Fridrich Loeffler Institute Tuebigen)
AS-M.5	Cutaneous angiosarcoma derived LEC	Kindly provided by Vera Krump-Konvalinkava (Institute of Pathology, Johannes Gutenberg Univeris Mainz)
HeLa-CEACAM1	Human cervix carcinoma transfected with CEACAM1	Kindly provided by Dr. Bernhard B. Singer (Institute of Anatomy, University Hospital Essen)

Table 7: Cell lines used in the study

The Dulbecco´s modified Eagle's medium (Gibco) was supplemented with 10% (v/v) heat-inactivated FCS (PAA), 2 mM L-glutamine (Gibco), 100 Lµ/ml penicillin (Gibco),100 µg/ml streptomycin (Gibco) and pre-warmed to 37^{o}C prior to use. Fetal bovine serum was heat inactivated at 65°C for 30 minutes before use. The cells were grown in 5% CO_2 humidified atmosphere at 37^{o} C.

2.2 Methods

2.2.1 Protein analysis

2.2.1.1 Extraction of protein

Adherent cells were removed from the flask by 0.05% trypsin-EDTA transferred into 3 ml 10% FCS medium. Both adherent and suspension cells were centrifuged for 3 minutes at 250 x g, and then washed with cold PBS. The cell pellets were resuspended in RIPA-lysis buffer which contains protease inhibitor (1:100), vortexed and incubated on ice for 10 minutes. The samples were sonicated (Bundelin sonopuls, Germany) at 5 x 10% cycles for 5 seconds pulses. Then, the samples were centrifuged for 10 minutes at 18620 x g at 4°C. The supernatant were collected and kept at -20°C until further analysis. The protein concentration was determined using one of the following methods, BCA Protein Assay, Bradford Assay or Fluitest USP kit.

2.2.1.2 BCA protein assay- Pierce

Protein concentration was measured utilizing the bicinchoninic acid (BCA) method. The standard curve was determined according to manufacture's instructions. The BSA (Bovine Serum Albumin) was diluted in 0.9% NaCl or 50 mM Tris buffer. The standards and appropriate diluted samples (1:5) were measured in duplicates. The working reagent (WR) was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B) while it's total volume was calculated using the formulae below.

(# Standards + # unknowns) X (# replicates) X (volume of WR per sample = total volume WR required.

1 ml of working reagent was added to cuvettes, and was incubated at 37°C for 30 minutes, then allowed cooled at room temperature before the absorbance measurements at 562 nm. Sample protein concentrations were estimated applying the BSA standard curve.

2.2.1.3 Micro BCA protein assay- Pierce

The Kit was utilized to determine low concentrated protein samples 0.5 µg/ml - 20 µg/ml. The kit uses concentrated reagents and its performance needs an incubation time of 1 hour at 60^{o}C. The working reagent was prepared by mixing 25 parts of MicroBCA Reagent MA, 24 parts Reagent MB with 1 part Reagent MC. The protein concentrations were determined using bicinchoninic acid (BCA) according to the manufacturer's manual. Sample protein concentrations were estimated applying BSA standard curve.

2.2.1.4 Bradford assay-BioRad

Bradford´s method was performed as described by (Bradford, 1976). BioRad Protein assay kit was used and bovine IgG (gamma globulin) was used as the standard, dilute in PBS from 0.2 mg/ml to 1.5 mg/ml. The samples were diluted in PBS (1:10) and 10 µl was applied the protein concentration determination. The working reagent was prepared by mixing 1 part of reagent and 4 parts of H_2O. From the working reagent 500 µl was used. The absorbance was measured at 595 nm and protein concentration determined applying the IgG standard curve.

2.2.1.5 Fluitest USP kit

Fluitest test USP kit from Biocon Diagnostics, Germany was used for the protein determination. In this method pyrogallol-red-molybdate complexes are bound to proteins. The binding causes a shift of absorbance peak. The increase of absorbance is directly proportional to the protein concentration. The standard curve was prepared by adding 10 µl of reagent 4 into two wells in an ELISA plate. Then 10 µl of protein samples were transferred into two different well, subsequently 240 µl of reagent 1 was to all wells. For the blank 250 µl of reagent 1 was transferred into different wells. The plate was then incubated at room temperature for 10-30 minutes before the absorbance of the samples were measured in an ELISA reader at 620 nm. The assay was performed in triplicates.

2.2.1.6 Tricine polyacrylamide gel electrophoresis

Tricine-SDS-PAGE developed by (Schagger and von, 1987). In this technique tricine replaces glycine in the running buffer resulting in more efficient stacking and disstacking of low molecular weight proteins (5-20 kDa) and high resolution of smaller proteins. Tricine ions migrate ahead of proteins thus improving the separation of small size proteins at lower acrylamide concentrations than in the SDS-PAGE systems. Proteins above 30 kDa are disstacked within the stacking gel thus helping in smooth migration of these proteins in to the separation gel. Tricine does not interfere with the amino acid sequencing. In addition, to good transfer efficiency, the tricine system has a lower pH which minimizes unwanted protein modification. Tricine polyacrylamide gels were prepared using the following protocol.

Separation gel

Solutions	9%	11%
30% Arcylamide/Bisaryclamide	2.25 ml	2.75 ml
Tricine gel buffer	2.5 ml	2.5 ml
H_2O	2 ml	1.5 ml
Glycerine	1 g	1 g

APS 10% (w/v)	37.5 µl	37.5 µl
TEMED	3.75 µl	3.75 µl

Table 8: Reagents and buffer for preparing tricine separation gel

Stacking gel

Solutions	4%
30% Arcylamide/Bisaryclamide	0.83 ml
Tricine gel buffer	1.55 ml
H_2O	3.8 ml
APS 10% (w/v)	50 µl
TEMED	5 µl

Table 9: Reagents and buffer for preparing tricine stacking gel.

In most cases 9% and 11% of Tricine gels were used in combination with 4% of stacking gel buffer to allow optimum separation. High percentage gels were used because they separate large molecular weight proteins better than low molecular weight proteins. The cassettes were filled up to approximately 5 cm with the separating gel mixture, then immediately the stacking gel was poured on to the top. A 10 or 15 comb was inserted to create the pockets for sample loading. The gel was allowed to polymerize for 30 minutes, at room temperature. After polymerization, the comb was carefully removed. The cassettes containing the gels were vertically inserted into the electrophoresis chamber and filled with tricine running buffers. For tricine gel electrophoresis two different running buffers were used. The middle chamber between the gels was filled with cathode buffer while the outer chamber with anode buffer. The samples were mixed well with equal volume of 5X laemli sample buffer to give a final concentration of 2.5X with subsequent reduction by addition of 75 mM of Beta-merptoethanol. Then the samples were boiled at 95^{o} C for 10 minutes. Prestained protein marker from Fermentas was used to estimate molecular size of separated proteins. Then the prepared samples were loaded using Hamilton syringe. The gels were run first at 45°C to pull down the samples to the separation gel slowly and then increased to 120 volts. The gel was then removed from the cassette and proteins transferred on a nitrocellulose membrane.

2.2.1.7 SDS–Polyacrylamide gel electrophoresis

Protein samples were separated in a denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system (Laemmli, 1970). Most case 10% gels were used. To prepare the samples for SDS-PAGE separation, first the samples were reduced by addition

of laemmli sample buffer in 1:1 v/v (from 5X stock) and subsequent reduction by addition of 75 mM β-mercaptoethanol. Then the samples were heated for 10 minutes at 95^{o}C.

SDS-PAGE cassettes from Bio-Rad were used for gel preparation and the gels were prepared as follows.

Separation gel

Solutions	10%
30% Acrylamide/ Bis-Arcylamide	3 ml
Separation Buffer	2,25 ml
H_20	3.75 ml
APS 10% (w/v)	45 µl
TEMED	4.5 µl

Table 10: Reagents and buffer for preparing SDS-PAGE separation gel.

Stacking gel

Solutions	4%
30% Acrylamide/ Bis-Arcylamide	0.4 ml
Separation Buffer	0,75 ml
H_20	1,85 ml
APS 10% (w/v)	12 µl
TEMED	3 µl

Table 11: Reagents and buffer for preparing SDS-PAGE stacking gel.

The cassettes were filled up to approximately 5 cm with the separating gel mixture and a thin layer of water was added to flatten the top of the separation gel also to prevent its evaporation. Then it was allowed to polymerize for 30 minutes at room temperature. After polymerization water is poured off and immediately the stacking gel mixture added on top. The sample preparation and the loading were done in the same ways as in tricine gel electrophoresis. SDS running buffer was used. After the separation was completed the proteins were transferred in to the nitrocellulose paper.

2.2.1.8 Western blotting

After efficient separation of proteins inside Tricine-SDS-PAGE or SDS-PAGE, proteins were transferred into nitrocellulose membrane (Millipore) using wet transfer inside electric field (Western blotting). For blotting nitrocellulose membranes were cut in size as 7x9 cm. The nitrocellulose membrane, sponges and Whatman filter papers were first soaked in blotting buffer shortly before placing. The Western blot chamber was arranged as follows. To

assemble transfer set the thick sponge was placed on the anode side of the transfer unit followed by three Whatman filter papers, then nitrocellulose membrane. And the gel was placed on it, covered with two Whatman papers and a thin sponge on the cathode side. Air bubbles were removed from the whole transfer set by rolling a glass over it from left to right. For transferring of proteins from gel to nitrocellulose membrane, equipments as Mini Trans-Blot-System obtained from BioRad were used according to the manufacturer's recommendations. The cassettes were placed in the chamber and a cold pack was put beside and filled with pre-chilled 1x blotting buffer. Electrophoretically transfer was performed 1 hour using 100 voltages. Quality of the gel was confirmed by staining the membrane with ponceau red dye solution. The blot was incubated for 1-2 hours at room temperature or at 4^{o}C overnight in 3% non-fat dry milk dissolved in TBS buffer to block the remaining surface of the membrane to prevent nonspecific binding of the detection antibodies during subsequent steps. Then, the memrane was incubated with primary antibody (8 µg/ml) 3% milk/TBS solution at 4°C over night or 2-4 hours at room temperature. After incubation, the membrane was washed three times in TBS buffer with five minute interval to remove unbound antibodies. Then, secondary horseradish peroxidase (HRP) conjugated antibody solution (1:5000) was added for 1-2 hours at room temperature diluted in 1.5% non-fat dry milk power in TBS buffer. After three washing steps, the membrane was developed by chemiluminence and visualized by LAS 3000 Imager (Fiji- film). All incubations were performed on a shaker.

2.2.1.9 Immunoprecipitation

The cellular extracts, cell culture supernatants, feces and urine probes were incubated for 1 hour at 4°C with 40 µl of protein G sepharose 4 Fast Flow beads (GE Healthcare) for preclearance. Then, the samples were centrifuged for 1 minute at 800 x g and the supernatant transferred into new tubes. After centrifugation, 2-10 µg of monoclonal antibodies against CEACAM1 or polyclonal rabbit anti-Human carcinoembryonic antigen (DAKO) was added and incubated for 2 hours at 4°C under permanent rotation. Then, 60 µl of protein G sepharose beads were added to the samples and further incubated at 4°C overnight under permanent rotation. The antigen-antibody-protein G beads complexes were pelleted by centrifugation at 400 x g for 1 minute, washed three times with 1x PBS. Then, added in 60 µl of 2.5x Laemli sample buffer containing 75 mM β-mercaptoethanol, and boiled at 98^{0}C for 10 minutes. Subsequently the samples were centrifugated at 2300 x g for 5 minutes and the supernatant transferred into fresh tube. Fractions of the probes were loaded on an 11% tricine gel electrophoresis followed by transfer onto nitrocellulose membrane.

2.2.1.10 PIERCE direct immunoprecipitation Kit.

The immunoprecipitation was performed using the PIERCE DIRECT IP KIT (Thermo scientific). The beads were first coupled with 10 µg of mouse monoclonal anti-CEACAM1

antibody according to manufacture instructions. The coupling elution supernatants were kept for controling of coupling efficiency by Western blot and ELISA. After, coupling A549 cells were lysed using the lysis buffer provided in the Kit. The protein concentration in the lysate was determined using Fluitest USP Kit. 585 µg of cell lysates was transferred into two tubes one contained the anti-CEACAM1 coupled beads while the other Pierce control agarose resins. The samples were incubated overnight at 4^{o}C under permanent rotator. Next the antigen-antibody-beads complexes were pelleted by centrifugation at 400 x g for 1 minute, washed three times with PBS. The proteins were eluted using elution buffer provided in the kit and immunoprecipitation flow through were kept for controlling of the efficiency of immunoprecipitation by Western blotting. Then the proteins were separated via 9% tricine gels, blotted and subsequently detected for CEACAM1.

2.2.1.11 Protein deglycosylation

Deglycosylation was performed using PN Gase F and additional reagents as provided by the supplier (New England Biolabs). The use of enzyme PNGase F is the most effective method of removing virtually all N-linked oligosaccharides from glycoproteins. The enzyme cleaves between the innermost GlcNaC and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins (Maley et al., 1989).

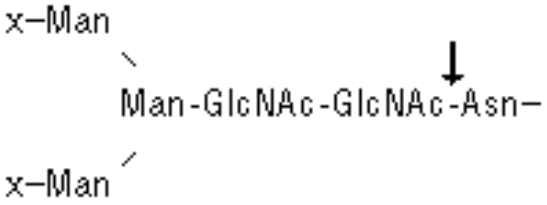


Figure 4: Chemical reaction illustrating the cleaving site of PNGase enzyme on the oligosaccharides.

PNGase F hydrolyzes nearly all types on N-glycan chains from glycopeptides/proteins. [X=sugar(S)]. Obtained from New England Biolabs.

First immunoprecipitation was performed to precipitate CEACAM1 from various cells (A549, T102/3, HT29 and AS-M.5) and from 40-50 ml of urine as described above. The antigen-antibody-beads complexes were suspended in 55 µl of denaturing buffer which contained 450 µl deionized H_2O, 60 mM DTT, 50 µl 10x glycoprotein denaturing buffer supplied by manufacturer and subsequently boiled at 98^{o}C for 10 minutes. Then, the beads were centrifuged at 2300 x g for 5 minutes. The supernatant containing eluted proteins was carefully transferred into new tube. Samples were divided into two fractions for non-deglycosylated and deglycosylated probes. To prepare the total final volume of 30 µl probes the following master mix was prepared as shown below (Table 12).

Components	Non-deglycosylated	deglycosylated
Protein probes	22 µl	22 µl
10x G7 reaction buffer	3 µl	3 µl
10% NP 40	3 µl	3 µl
H^2O	2 µl	-
PNGase	-	2 µl
Total volume (per probe)	30 µl	30 µl

Table 12: compostion of deglycosylation reaction

The samples mixed by vortexing and centrifuged briefly. For the digestion the samples were placed for 1 hour at 37^{o}C. Reaction was stopped by addition of 5x SDS buffer and boiling at 98^{o}C for 10 minutes. Then, the samples were subjected to Western blotting as above described.

2.2.1.12 Coomassie staining

Coomassie Blue is a disulfonated triphenylmethane textile dye which was introduced for protein detection (Fazekas de St et al., 1963). The dye can be found in two modifications of Coommassie G-250 (greenish tint, dimethylated form) or R-250 (reddish tint). In acidic solution the dye sticks to the amino groups of the proteins by electrostatic and hydrophobic interactions. The technique has a detection limit of 50-100 ng. The staining may be done in presence of alcohol (methanol, ethanol or isopropanol up to 45%) and acid (acetic acid, trichloroacetic acid). The distaining of the gel is performed with similar solution but which do not have the dye (Chrambach et al., 1967; Weber and Osborn, 1969). In this technique the proteins have a higher affinity to the dye than the gel matrix, therefore distaining can be carried out until the background is low and the protein bands are clearly visible.

The gels with separated proteins were placed into coomassie staining solution containing coomassie blue G-250 (sigma) for 2 hours at room temperature or overnight at 4^{o}C. Then the gels were incubated gels in destaining solution for 2-4 hours at room temperature or until protein bands were clearly visible.

2.2.1.13 Silver staining

After the gel electrophoresis the tricine or SDS-PAGE gels were further stained with silver staining technique which has higher sensitivity than Coomassie staining. The technique provides a very sensitive tool for protein visualization with a detection level down to the 0.3-10 ng level (Switzer, III et al., 1979). The mechanism underlying silver staining of protein or peptides in gels are well understood. Protein detection depends on the binding of silver ions

to the amino acid side chains mainly the sulfhydril and carboxyl groups of proteins (Switzer, III et al., 1979; Merril et al., 1981a) then followed by reduction to free metallic silver (Rabilloud, 1990). The protein bands are visualized as spots where the reduction occurs and this is based on the difference in oxidation-reduction reaction between the gel's area occupied by proteins and the unoccupied regions.

The gel was placed into a tray containing appropriate volume of fixation buffer for 2 hours at room temperature or at 4^{o}c overnight to restrict protein movement from the gel matrix and to remove interfering compounds present in the gel. Fixation buffer contains formaldehyde thus, introducing the chemical modification into proteins by causing the cross-linking of two lysine residues within protein chains. Fixation buffer was discarded to a chemical compatible container with a sealed lid and labeled clearly because it can cause severe health problems if swallowed, inhaled or absorbed through the skin. The gel was washed in 20% ethanol for 20 minutes at room temperature. The wash step was repeated three times.

Alternatively, coomassie stained gels were silver stained to visualize bands which could not be detected. Here no additional fixation step was needed. After washing, enough volume of sensitizing solution was added and incubated for 2 minutes by gentle shaking at room temperature; this further increased the sensitivity and the contrast of the staining. Then, the gel was washed twice one minute each in deionized water. Then pre-cooled silver staining solution was added to the gel, incubated for 20 minutes at room temperature to allow the silver ions to bind to proteins. The staining solution was not poured directly on the gels at it may result in unequal background, in this it was added to the corner of the tray. After staining was completed, the staining solution was discarded and the gel rinsed twice with a large volume of deionized water for 20-60 seconds to remove the excess of unbound silver ions. Washing the gel for more than 1 minute was avoided because it would remove the silver ions from the gels resulting in decreased sensitivity.

The gel was rinsed shortly with developing solution and immediately a fresh portion of developing solution was added, incubated for 2-5 minutes. As soon as the desired intensity of the bands was reached the reaction was stopped by adding of stopping solution. The gel was documented in Fiji gel documentation system.

2.2.1.14 NitroBlue staining

The technique allows the negative staining of proteins (Taketa et al., 1986). The gel matrix is stained blue by photoreduction of Nitro Blue Tetrazolim (NBT) into formazan while the protein bands remain unstained. The method is much more sensitive that Coommassie with a detection limit of 10-100ng.

Immediately after gel electrophoresis, the gel was rinsed in deionized water, and then placed in a Tris buffer containing reduced glutathione-GSH (Sigma).Then the gel was incubated for 15 minutes at room temperature. After incubation the gel was rinsed with deionized water

before staining in Tris containing nitroblue tetrazolium. The entire gel was stained dark blue after incubation of 2 minutes at room temperature, except for unstained protein bands. The staining was halted by rinsing the gel in deionized water.

2.2.1.15 Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Two-dimensional electrophoresis (2D-electrophoresis) was first introduced by (O'Farrell, 1975) and (Klose, 1975). The technique separates protein mixtures in the first dimension according to their isoelectric point (pI) and in the second dimension by their molecular weight (MW) as shown in the figure below.

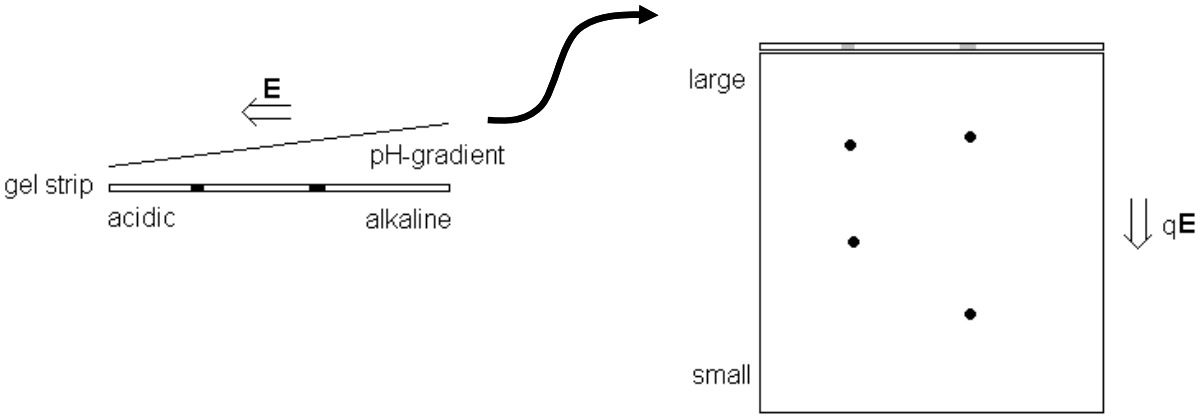


Figure 5: Cartoon of 2D-SDS-PAGE.
Proteins are separated in two dimensions horizontally by iso-electric point (pI) and vertically by molecular weight (MW) and each spot corresponds to a single protein.

2.2.1.16 Urine collection

Urine samples were collected from healthy male and female individuals. The first morning urine specimens were collected, a portion analyzed as fresh while the rest stored at -20°C (for a longer time than 2 months at -80°C) until use. Sodium azide (NaN_3) was added to inhibit bacterial growth. Before any analysis was performed the samples were centrifuged at 2300 x g for 10 minutes to remove cell debris.

2.2.1.17 Preparation of urine samples for 2-DE-PAGE

The urine samples were first purified by dialysis in order to remove salts and to adjust the pH. The dialysis was performed against dialysis buffer usually 1x PBS, using 10 kDa molecular weight cut-off (10K MWCO). 100 ml urine was dialysed against 1000 ml PBS. Dialysis was carried out for 24 hours at 4^oC with 3 times. Subsequently urine sample were applied to immmunprecipitaion and Western blot.

2.2.1.18 Measurement of the acidification in the urine samples

Immunoprecipitation needs pH 7.3 because this increases antibody-antigen capture by protein G beads. Thus, acidification was determined utilizing cell debris-free urine for

estimating the pH value by the color change of the tracers in the urine and the pH-indicator strips pH 5.2-7.5 (Merck, Darmstadt, Germany). Urine was considered as acidified if the pH decreased below 7.0 and was adjusted using 10x PBS.

2.2.1.19 Sample elution

The sepharose beads which contain precipitated proteins were washed 3 times with precold PBS. Then, 100 µl of rehydration buffer 2 (Biorad) prepared by addition of 75 mM β-mercaptoethanol was added, and incubated for 30 minutes at 5°C in a thermo-mixer rotating at 5 x g. After elution the beads were centrifuged for 2 minutes at 800 x g and the supernatant transfer into a new tube. Another 100 µl of rehydration buffer 3 which contained 0.05% (w/v) DTT was added then incubated and centrifuged as before. The supernatant obtained were pooled for further analysis.

2.2.1.20 Rehydration

The PROTEAN Isoelectric focusing (IEF) cell with integrated power supply was used for rehydration and IEF protocols. The eluted protein samples were loaded onto IPG strips in IEF focusing tray for rehydration and first dimensional separation. IPG strip gels were placed side down in the channel of a focusing or rehydration tray that contains the sample in rehydration solution. For urine samples mostly 11 cm nonlinear IPG strips with 3-10 pH range were used. Strips were covered with 2 ml of mineral oil to prevent evaporation which causes the urea to precipitate as it becomes more concentrated. IPG strips with sample in a focusing tray were active rehydrated by running of IEF cell under low voltage as 50 volts overnight.

2.2.1.21 First dimensional separation: Isoelectric focusing (IEF)

After the strips have rehydrated the ends of each strip was lifted and wet electrode wicks were inserted between the strip and the electrodes. Strips were covered with mineral oil before starting the focusing run to prevent evaporation and carbon dioxide absorption during focusing. Isoelectric focusing was performed at voltage linearly increased from 150 to 5000 V during the first 5 hours, followed by 8000 V for a total of 70000 Vh/h

Temperature 20˚C

Current max. 0.05 mA per strip

Sample volume 200 µl for 11 cm strip, 300 µl for 17 cm strip

Step	Voltage	Time / Volt-hour
1	150 V	30 minutes. rapid
2	300 V	60 minutes. slow
3	1000 V	60 minutes. slow
4	1500 V	60 minutes. slow

5	2000 V	60 minutes. slow
6	3000 V	60 minutes. slow
7	5000 V	60 minutes. slow
8	8000 V	4 hours slow
9	8000 V	70000 Vh linear
10	500 V	3 h linear

Table 13: Applied voltage steps for IEF

2.2.1.22 Equilibration

This process reduces disulfide bonds and alkylates the resultant sulfhydryl groups of the cysteine residues. Rehydration/equilibration trays sized for each size strip were used for equilibration. Each strip was moved to equilibration tray and the channels were filled with the equilibration buffers. Strips were first incubated in DTT equilibration buffer-I that reduces sulphydryl groups for 15 minutes at room temperature, and then the channels were refilled with iodacetamide equilibration buffer-II which alkylates sulfhdryl groups and incubated for 15 minutes at room temperature. For the strips with 11 cm length, 3 ml of each equilibration buffers were used. After equilibration, IPG strips were removed and embed into the prepared second-dimension gel.

2.2.1.23 Second dimensional separation

The second dimension electrophoresis of isoelectric focused proteins was carried out on pre-casted polyacrylamide gels obtained from BioRad (Criterion Precast Gel 10% Tris-HCl, 1.0 mm). The equilibrated IPG gel strips were placed on top of the polyacrylamide gel and overlaid with 2 ml 0.5% melted agarose prepared in SDS-PAGE running buffer. A small amount of Bromophenolblue was added to the agarose overlay to track the ion front during the run. The SDS gel running was performed with a voltage setting of 60 V for 15 minutes and then at 120 V until the end. BioRad Dodeca Cell which accommodates 12 gels per run was mostly used for proteomics analyses. To estimate the molecular mass of each spot, marker proteins were placed on a filter strip left to the strip on the gel. Polyacrylamide gels were stained with silver and/or Coomassie Brillant Blue (BioRad) according to the procedures described previously (Merril et al., 1981a).

2.2.1.24 Protein detection

To visualize proteins after separation in 2-DE gels it is necessary to use an adequate staining method. The enormous variation in characteristics and abundance of the individual proteins put high demands on the staining technique. The most important requirements are high sensitivity, high linear dynamic range, high reproducibility and compatibility with mass spectrometry. Major developments of protein staining methods have occurred during the last

few years. Since silver staining is the most sensitive non-radioactive detection technique and the costs for reagents are relatively low when compared to fluorescence techniques, it is still widely used. There are presently more than 100 different modifications of the original silver staining protocol that was introduced by (Merril et al., 1981a). In this study silver staining, coomassie blue staining Western blotting methods were used to visualize the protein spots.

2.2.1.25 Concentration of urinary proteins by acetone precipitation

Acetone precipitation was performed on urine sample to precipitate proteins present in the urine. 400 µl of urine was transferred into a 2 ml microcentifuge tube containing 1.4 ml pre-chilled acetone. The samples were mixed by vortexing, and incubated overnight at -20°C. The precipitates were obtained after centrifugation 10 minutes at 18620 x g. Then the pellets were air dried. However the pellet was not completely dried to allow resuspension complete. Subsequently, the pellet was resuspended in 100 µl PBS and stored at -20°C until further use.

2.2.1.26 Concentration of urinary proteins via centricon columns

Urine sample of 2 ml volume was applied onto a Centrifugal Filter Column (Amicon) to remove molecules smaller than 5 kDa such as urea, electrolytes, and salts and to increase the protein concentration of the urine samples. The sample was centrifuged at the speed of 800 x g at 4°C. The pass through was discarded while the up portion kept for further analysis on gel electrophoresis.

2.2.1.27 Separation of hydrophilic and hydrophobic fractions via ultracentrifugation

Ultracentrifugation allows elimination of membrane vesicles and cell granules by extremely high speed. A volume of 10 ml urine samples was collected and centrifuged at 4000 x g for 10 minutes to removes any cells debris in it. Subsequently, a fraction of 1.5 ml was transferred into an Eppendorf tube and labeled as “before ultracentrifugation”. The rest of the fraction was transferred into ultracentrifugation tubes.

Alternatively, 20 ml of cell culture supernatant from different cell lines were collected and centrifuged at 4000 x g for 5 minutes to removes any cells debris. Then, 8 ml were transferred into different tubes and labeled as “before ultracentrifugation” this contained both the hydrophilic as well as hydrophobic fractions. Concurrently, we transferred 9 ml in ultracentrifugation tubes. The samples in the ultracentrifugation tube were then centrifuged at 41823 x g in a Sorvall SA-600 rotor (Thermo Scientific) for 2 hours at 4°C. Thereafter 1 ml of urine sample and 8 ml of cell culture supernatant was carefully transferred into fresh tubes. All sample fractions were then analyzed by ELISA and immunorecipitation using the protocol described above.

2.2.1.28 Phase separation assays

10 ml of Triton X-114 was transferred into a 50 ml tube, and 40 ml of 1x PBS containing a drop of Bromophenol blue was added. The tube was mixed well by vortexing and rested at room temperature for 1 hour. Then, the tubes were centrifuged at 4000 x g for 30 minutes keeping without break gradient to avoid destruction also to separate the lipophilix TX-fraction with hydrophilic fraction. The PBS was carefully sucked off. 500 µl of concentrated Triton X-100 was transferred into a tube containing same volume of cell debris-free urine. Then, the sample was mixed by vortexing and incubated under permanent rotation at room temperature for 1 hour. Afterwards the tube was centrifuged at 4000 g for 5 minutes .The upper hydrophilic and lower hydrophobic fractions were transferred into different tubes. The concentration of CEACAM1 in each fraction was analyzed by sandwich ELISA.

2.2.1.29 Sucrose gradient separation

Cells were incubated with 1 ml DMEM medium having 10 µg/ml of anti-CEACAM1 monoclonal antibody (18/20) for different time intervals from 5 minutes, 15 minutes, 30 minutes, 45 minutes, and 1 hour. As control no mouse antibody was applied to the samples. The samples were lysed in 200 µl PIRA buffer utilizing the protein extraction protocol (3.2.1.1). The gradient was formed by layering from the bottom to the top of a 10 ml tube 1 ml each of 48%, 35%, 25%, 17%, 10%, and 1% sucrose (sucrose was diluted with PIRA buffer). This gradient was overlaid with 200 µl of cell lysate. Samples were then centrifuged at 235000 x g in a type 45 Ti rotor (Beckmann) for 18 hours at 4^{o}C. After centrifugation, sample fractions of 516 µl each were carefully collected from top to bottom. Fractions were separated by 9 % Tricine gels electrophoresis.

2.2.2 Immmunological methods

2.2.2.1 Solid phase ELISA -Enzyme-linked immunosorbent assay

This type of ELISA the antigen is directly immobilized to the ELISA plate.

1. Antigen: 100 µl of appropriate sample was added into NUNC-Immuno plate (Thermo fischer scientific Roskilde, Denmark) and incubated for atleast 2 hours at room temperature or alternatively at 4°C overnight. Usually urine samples were diluted 1:2 with 1.5% blocking buffer, cell lysate 1:50 and supernatants were not diluted. After incubation the sample was flipped off, and then 300 µl of blocking buffer (3% BSA-PBS) was added. Then incubated the plate for 1-2 hours at room temperature then followed by washing steps. The washing was done by filling the wells with 150 µl of PBS and flicking the washes out.
2. Detection antibody: Then the plate was incubated with 100 µl anti-CEACAM1 monoclonal antibody (10 µg/ml) diluted in 1.5% BSA-PBS for 2-4 hours at room temperature or overnight at 4^{o}C followed by washing steps. For biotinylation assay the plate was incubated with 100 µl biotin-coupled anti-CEACAM1 monoclonal antibody (10 µg/ml) diluted in 1.5% BSA-PBS.
3. Labeling: After, washing the plate was incubated with 100 µl horseradish peroxidase-conjugated goat anti-mouse IgG diluted in 1.5% blocking buffer. The plate was then washed and completely dried by flipping the plate before the substrate was added.
4. Substrate: Enzymatic reaction was developed by adding 150 µl of TMB substrate (Sigma) and the reaction product stopped after 15-30 minutes by addition of 20 µl of 2M H_2SO_4. Absorbance at 450 nm was measured in a microplate reader (Tecan Germany). The standard curve and blank were run in each plate to ensure accuracy and for accurate quantitative results were always compared with the signal of samples against those of standard curve.

This ELISA technique is cheap and fast although it has some limitations associated with sensitivity, specificity and accuracy due to the factors concerning the binding of antigen to the solid matrix. These can be greatly increased by the use of another type of ELISA referred to as sandwich ELISA.

2.2.2.2 Sandwich ELISA

This type of ELISA is more specific and sensitive than the solid phase ELISA because the antigen is measure between two antibodies referred to as capture and detecting antibodies, which can be either monoclonal or polyclonal. The technique is also more accurate and if purified antigen standard is available, the assay can determine the absolute amount of antigen in an unknown sample.

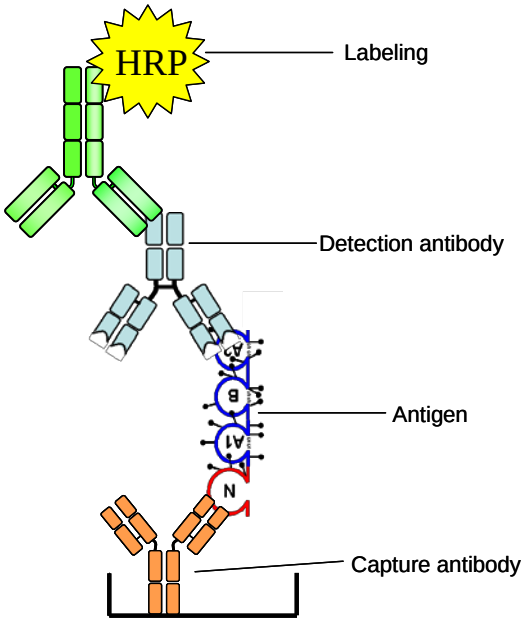


Figure 6: A cartoon illustrating Sandwich ELISA.

The antigen is sandwich between the capture antibody on the bottom of the plate and the detection antibody.

In our studies it was performed as follows:

1. Capture antibody: NUNC-Immuno plate (Thermo fischer scientific Roskilde, Denmark) was coated overnight at 4^{o}C with 5 µg/ml of the capturing antibody polyclonal rabbit anti-human carcinoembryonic antigen (DAKO) or with monoclonal antibody B3 diluted in PBS. After incubation capture antibody solution was removed by flicking the plate over a sink. Then the plate was blocked with 300 µl of blocking buffer (3% BSA in PBS) for 1-2 hours at room temperature followed by washing steps. The washing was done by filling the wells with 150 µl of PBS and flicking the washes out.
2. Antigen: Then, 100 µl of appropriate sample was added and incubated the plate for atleast 2 hours at room temperature or alternatively at 4°C overnight. Usually urine samples were diluted 1:2 with 1.5% blocking buffer, cell lysate 1:50 and supernatants were not diluted. Afterwards the plate was washed three times with 150 µl of PBS
3. Detection antibody: Then the plate was incubated with 100 µl anti-CEACAM1 monoclonal antibody (10 µg/ml) diluted in 1.5% BSA-PBS for 2-4 hours at room temperature or overnight at 4^{o}C followed by washing steps. For biotinylation assay the plate was incubated with 100 µl biotin-coupled anti-CEACAM1 monoclonal antibody (10 µg/ml) diluted in 1.5% BSA-PBS.
4. Labeling: After washing, the plate was incubated with 100 µl horseradish peroxidase–conjugated goat anti-mouse IgG diluted 1:5000 in 1.5% blocking buffer. The plate

was then washed and completely dried by flipping the plate before the substrate was added.

5. Substrate: Enzymatic reaction was developed by adding 150 µl of TMB substrate (Sigma) and the reaction product stopped after 15-30 minutes by addition of 20 µl of 2M H_2SO_4. Absorbance at 450 nm was measured in a microplate reader (Tecan Germany). The standard curve and blank were run in each plate to ensure accuracy and for accurate quantitative results were always compared with the signal of samples against those of standard curve.

2.2.2.3 Detection of CEACAM1 expression by flow cytometry

To analyze the surface expression of CEACAM1 in different cell types flow cytometry analysis was performed. Adherent cells were collected by the EDTA-Typsin method (3.2.1.1) while PMN were directly utilized to FACS procedure. Up to 0.5 $x10^6$ cells, were incubated with CEACAM1 monoclonal antibody 18/20 (10 µg/ml) in FACS buffer (PBS with 3% FCS) for 1 hour on ice. The cells were washed 3 times with 300 µl of FACS buffer, vortexed gently and centrifuged by 250 x g for 3 minutes at 4^o C. The wash buffer was removed by flipping the tube once and touching a soft filter paper. Then, the cells were incubated for 30 minutes with 100 µl of secondary flourescein isothiocynate (FITC) conjugated antibody diluted 1:50 in FASC buffer. For negative controls cells were stained with secondary FITC antibody or with isotype matched control immunoglobulin. This was followed by washing steps. Cells were resuspended in 200 µl of FACS buffer prior to measurement. The samples were measured using a FASC calibur instrument (Becton Dickison) and the data were analyzed by Cell Quest software (Becton Dickison).

2.2.2.4 Absolute quantification of CEACAM1 on cell surfaces by flow cytometry

Flow cytometric quantification of the CEACAM1 expression was performed using DAKO QIFIKIT^R kit. The kit contains a series of beads, 10 µm in diameter and coated with different well-defined quantities of mouse monoclonal molecules (high-affinity anti-human CD5, Clone ST1, isotype IgG2a). The beads mimic cells with different antigen densities which have been labeled with primary mouse monoclonal antibodies, isotype IgG. The cells were stained with excess amount of monoclonal antibody 18/20 (10 µg/ml) diluted in FACS buffer. Negative controls were included of cells only stained with secondary FITC antibody. The samples were incubated on ice for 1 hour. 10 µl each of Set-Up Beads and Calibration beads were transferred into fresh tubes. Then, samples were washed 3 times with 300 µl of FACS buffer, vortexed gently and centrifuged by 250 x g for 3 minutes at 4^oC. The wash buffer was removed by flipping the tube once. Then, 100 µl of FITC conjugate diluted 1:50 in FACS buffer was, gently vortexed and incubated for additional 30 minutes on ice in the dark. After incubation the tubes were washed 3 times as described above. 200 µl of FACS buffer was

added for cytometry measurements. Set-Up Beads were used for the establishment of window of analysis and the fluorescence analysis was confirmed to the beads singled clear of debris as defined on a forward scatter versus side scatter dot blot. Without changing the analysis window the calibration beads and the samples were analyzed. The mean number of monoclonal antibodies molecules bound to cells was the calculated from the net mean fluorescence intensity by the use of the linear relation furnished by the standard curve.

2.2.2.5 Determination of apoptosis

Apoptosis (“programmed” cells death) is a physiological process by which unwanted cells are eliminated during development and other physiology processes. Apoptosis is defined by various characteristics changes such as cell shrinkage, increased cytoplasmic density, DNA fragmentation and formation of apoptotic bodies that contain nucleic or cytoplasmic material.
One of the events in apoptosis is the DNA fragments of approximately 185 bp (Wyllie et al., 1980). This cellular event can be analyzed by agarose gel electrophoresis which measures DNA fragmentation in nuclear extracts showing a typical DNA ladder shear (Compton and Cidlowski, 1986). Other improved methods visualize the DNA after Southern blotting with radiolabelled probes (Facchinetti et al., 1991) and 32P-labeled nucleic acid (Rosl, 1992). These techniques have several disadvantages; they are time consuming, requires a great number of cells, involves radioactivity in some more sensitive assays, and they are unable to determine the percentage of apoptotic cells or recognize the apoptotic cells in a heterogeneous cell population. DNA fragmentations can also be measured by fluoresces microscope after staining with a DNA-binding dye. This method is simple but it is not easy to discriminate between early and late apoptotic cells
To distinguish between early and late apoptosis flow cytometric analysis based on double-staining for Propidium Iodide (PI) and flourescein isothiocynanate (FITC) conjugated Annexin V can be used. An early event in apoptosis is rapid alternation in the organization of phospholipids leading to the flipping of the phosphatidyl serine (PS) from the inside surface to the out side surface of the plasma membrane. Annexin V-FITC can be used as flourenscent probe to label apoptotic cells. Annexin V binds to exposed PS, which appears in the outer leaflet of the plasma membrane in early apoptotic cells. The binding of Annexin V is calcium dependant process.

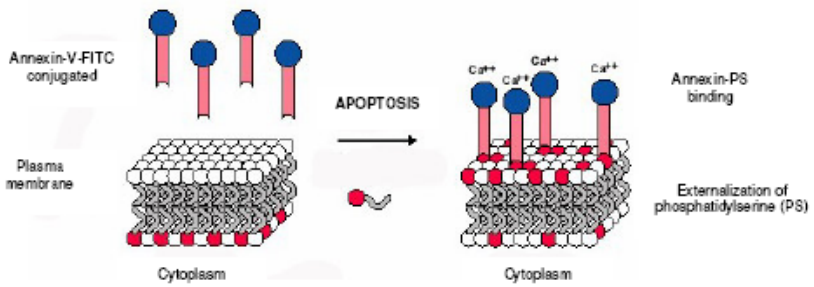


Figure 7: A cartoon representation of phospholipid flipping during apoptosis.

The picture shows the inversion of phosphatidylserine (PS) after induction of apoptosis and subsequent biding of Annexin V to the cell surface under well-difined cacium concentration (A and B). Obtained from www.bdbiosciences.com.

In the late apoptotic cells, the plasma membrane becomes permeable and allows uptake of PI, which intercalates into DNA. Thus, PI in conjunction with Annexin V-FITC staining allows discrimination between viable, early and late apoptotic cells as follows:

Viable cells

- Negative to Annexin V-FITC
- Negative to Propidium Iodide

Early apoptotic cells

- Positive to Annexin V-FITC
- Negative to Propidium Iodide

Late apoptotic/ Dead cells

- Positive to Annexin V-FITC
- Positive to Propidium Iodide

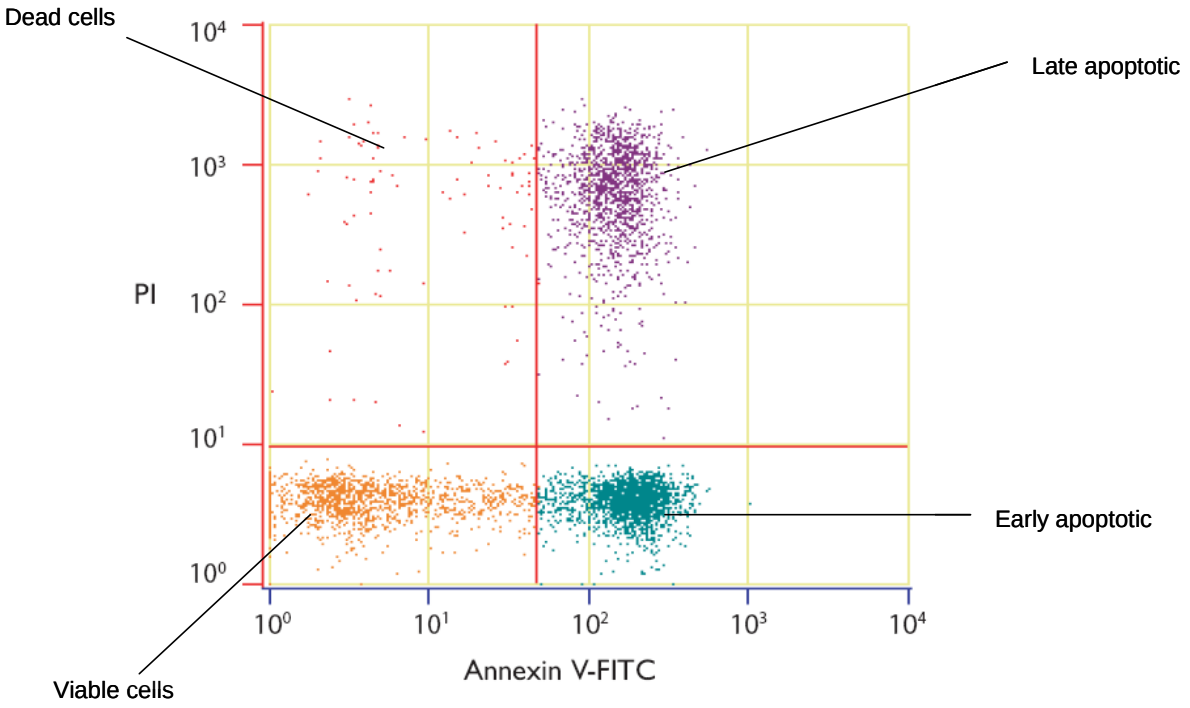


Figure 8: Representative dot plots of Annexin V-FITC/PI analysed flow cytometry.

A modified dot plot flow cytometic analysis of Jurket cells induced with camptothecin overnight at 37o C, stained with Annexin V-FITC and PI (From Cellular analysis business center, USA).

To determine apoptosis from different cell types flow cytometric analysis was performed after staining the cells using Annexin V-FITC and PI. Cells were cultured in presence and absence of following apoptosis inducers: actinomycin (30 µM), camptothecin (6 µM), cycloheximide (300 µM), dexamethasone (30 µM), etoposide (30 µM), and Fas-Ligand (0.2 µg/ml) for 24 hours. Old medium was replaced and the cells washed with PBS. Then, 1 ml DMEM which contained apoptosis inducer was transferred into the cells. A control was included in which apoptosis was not induced. Then the plate was incubated in the cell culture incubator for 24 hours. Up to 0.5 x10^6 cells were, incubated for 30 minutes in 50 µl DMEM containing Annexin-V FITC at concentration of 1:390. The cells were washed 3 times with 300 µl FACS buffer and centrifuged at 250 x g for 3 minutes. Then, DMEM containing PI at 1:120 concentrations was added and immediately the cells were analyzed by flow cytometry in a FASC Calbur instrument using Cell Quest software (Becton Dickison). A fraction of these cells were lysated and further analyzed by Western blot to compare CEACAM1 before and after apoptosis.

2.2.2.6 Leukocyte cells isolation

Human peripheral blood mononuclear cells (PBMC) and neutrophilic granulocytes [polymophonuclear neutrophils (PMN)] were isolated as follows

A total volume of around 40 ml of whole blood from healthy donors was collected in a tube containing NH2-Heparin (Sarstedt-Monovette). The blood was transferred into 50 ml tube under sterile condition. Then 1 ml of 6% dextran in DMEM was added into the blood, shaken gently to mix well and the air bubbles were removed my opening the lid then closing it immediately. Then sample was incubated for 45 minutes at room temperature to sediment erythrocytes. After sedimentation, the plasma was transferred into a fresh tube, centrifuged at 710 x g (centrifuge ROTINA 38 from Hettich zetrifugen, Germany) for 10 minutes at room temperature to pellet leukocytes. In case the solution was not clear 10 ml of 1x PBS was added and then centrifuged again as described above. The serum which does not contain blood cell and clotting factors was discarded while the pellet resuspended in DMEM medium. The leukocytes suspension was carefully layered onto a Ficoll-paque (GE Heathcare) which have density gradient of 1.075-1.077 g/ml. The sample was centrifuged at room temperature first at 710 x g for 3 minutes then the speed was increased to 1610 x g for 27 minutes without break. This resulted into two layers, the upper whitish layer of PBMC and PMN pellet containing some erythrocytes on the bottom of the tube. After centrifugation, the DMEM medium was removed and PBMC cells were transferred into a new tube. Then the Ficoll-paque was sucked off and the PMN pellet was gently mixed in 2 ml steril (Osmo-schock) H_20 for 20 seconds to lyze the remaining erythrocytes. The tube was filled up to 45 ml with DMEM medium. This step was repeated until all erythrocytes were lyzed. For the PBMC fraction the Ficoll-paque was completely removed by adding DMEM medium up to a volume for 45 ml. Both samples were centrifuged at 250 x g for 5 minutes at room temperature. PBMC and PMN cells were resuspended in 2 ml 10% FCS DMEM medium. The cells were counted. The purity of both cell fractions was verified by flow cytometric analysis of the granularity of the cells analyzing the side scatter (SSC).

2.2.2.7 Culturing and stimulation of PBMC

PBMC cells were isolated from blood of healthy donors by dextran sedimentation followed by Ficoll-paque as described above. Approximately 2 x 10^6 cells were transferred into three flasks having 1.5 ml culture medium containing phytohemagglutinin- PHA (Roche Diagnotics) (0.005 µg), anti-CD3 monoclonal antibodies(1 µg/ml) and anti-CD28 monoclonal antibodies (1 µg/ml). The samples were pipetted up and down to mix. As control a flask of non-stimulated PBMC cells was also included. Then, the flasks were incubated at 37^{o}C incubator overnight. In some assays stimulation kinetics was determined at different time points. After incubation, the stimulation was monitored by flow cytometric analysis. All samples were then analyzed by Western blotting using the protocol described previously.

2.2.2.8 Human monocytes differentiation to macrophages

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by dextran sedimentation followed by density gradient centrifugation using Ficoll-paque as described previously. Macrophages were obtained by culturing approximately 5 x10^6 PBMC cells in 10 ml DMEM medium containing 10 ng/ml of Granulocyte macrophage colony-stimulation factor (GM-CSF) and interleukin-3 receptor (IL-3 a colony stimulating factor) for 7 days. As control PBMC cells were cultured in absence of stimulating factors.

2.2.2.9 Co-Culture of macrophages and PMN

Cell undergoing apoptosis are usually recognized and swiftly ingested by macrophages. The clearance of apoptotic or senescent PMN by macrophages is a crucial process in events such as homeostasis, wound healing and tissue regeneration. To prevent damage of the surrounding tissues, PMN undergo apoptosis (Haslett, 1992) which leads to recognition and phagocytosis by macrophages. Inappropriate clearance of apoptotic cells may lead to autoimmune diseases and chronic inflammation (Rosen and Casciola-Rosen, 1999). Thus phagocytosis actively down-regulate inflammatory and immune responses further insuring safe clearance of apoptotic cells and unwanted proteins.

To determine what happens to CEACAM1 expressed on PMN, the PMN cells were co-cultured with macrophages. PMN cells were freshly isolated from heparinize blood of healthy donors by dextran sedimentation followed by Ficoll-paque as described previously. The mononuclear cells isolated by centrifugation on Ficoll-paque density gradient then differentiated to macrophages as described above. A flask having differentiated macrophages was taken and its supernatant transferred into fresh tube for future analysis. The flask was thoroughly washed with 1x PBS to remove undifferentiated monocytes. Then, the macrophages cells were overlaid with 10 ml DMEM media containing 20 x10^6 of freshly isolated PMN cells. As controls one flask was cultured with only macrophages and another with PMN cells only. Then, the flasks were incubated at 37 °C incubator for 24 hours. After the incubation, the cells and their supernatants were subjected to Western blotting as previously described.

2.2.2.10 Spontaneous apoptosis in PMN

Freshly isolated PMN were divided into two fractions each having 20 X10^6 cells. One portion was transferred in to an Eppendorf tube, pelleted down then frozen at -20^0C until further use. The rest of the cells were transferred in to a flask containing 10 ml DMEM medium. The flask was cultured in a cell incubator for 24 hours. Thereafter, the cells and supernatant were separately collected and further analyzed.

2.2.3 Protein and cell surface labelling

2.2.3.1 Roti^{R}-MagBeads COOH (ROTH) kit

The matrix of Roth–MagBeads-COOH consist of uniform superparamagnetic silica beads coated with surface carboxyls (-COOH) functional groups. The beads are of magnetic iron oxide with an average size of 1 µM. Protein binding was performed via functional amine groups. The beads were coupled with different concentrations of anti-CEACAM1 monoclonal antibodies 1-200 µg per 1 ml (3-4 X10^{9} beads / ml) of magnetic beads. The coupling was carried out according to manufacturer's instructions. All washing steps of beads were done via magnetic field. To monitor coupling efficiency the protein contain in reaction utilized protein determination methods (Bradford, BCA assay) was analyzed. The efficiency of coupling beads was also determined by Sandwich ELISA and flow cytometry before their use in immnunoprecipitations.

2.2.3.2 MagnaBind^{TM} Carboxyl Derivatised Beads (PIERCE) Kit

MagnaBind^{TM} beads can be used for affinity chromatography procedures to purify specific molecules from a complex mixture. The beads were supplied as aqueous suspension of magnetic iron oxide beads coated with carboxyl groups for covalent coupling of molecules using EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbiimide hydrochloride). EDC is a crosslinker that is reactive to amine and carboxyl functional groups. 1 ml of beads was washed with 1 ml PBS to remove storage buffer. In our studies anti- CEACAM1 monoclonal antibodies were diluted in conjugation buffer containing MES (N-Morholinoethane sulfonic acid) into different concentrations (1-200 µg) and transferred into the washed beads. The coupling was performed according to manufactures instructions.

2.2.3.3 MagnaBind^{TM} Amine derived beads.

The coupling of anti-CEACAM1 monoclonal antibodies was done according to the manufacturer's instructions. In short, 1 ml of beads was coupled with 2.5-10 mg/ml of anti-CEACAM1 monoclonal antibodies using BS^{3} as chemical cross linker. BS^{3} (5 Mm) was dissolved in PBS. The sample was incubation for 30 minutes at room temperature for coupling reaction. Then, the coupling reaction was stopped by addition of stop buffer (1 M Tris Ph 7.5). The quality of coupled beads was analyzed by sandwich ELISA and flow cytometry before they were used for immunoprecipitation.

2.2.3.4 Biotinylation assays

Biotin is a vitamin found in minute amount in every living cell. Biotin is a small molecule (244 dalton) by which it can be introduced to proteins, in most cased without significant altering their biological activities. In most cases proteins or cell molecules are biotinylated via amine fuctional groups by using biotinylation reagents N-hydroxysuccinimide (NHS) esters and N-

hydrosulfosuccinimide (sulfo-NHS) esters. In our biotinylation the following reaction was preformed

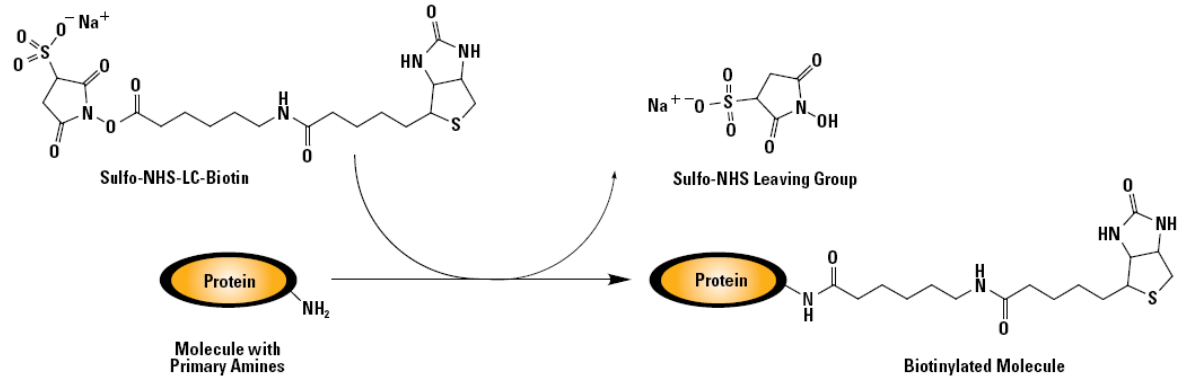


Figure 9: Chemical reaction illustration of protein biotinylation.
Obtained from Avidin-Biotin technical handbook –Thermo Scientific/ Pierce.

The cells were washed two times with PBS, followed by three washing with PBS which contains $CaCl_2$ (0.0901 mM) and $MgCl_2$ (0.493 mM) (Gibco). The cells were incubated for 1 hour on ice by gentle shaking in presence and absence of 0.5 mg/ml PBS-Biotin (EZ-link - Sulfo-NHS-LC-Biotin from Pierce) solution. After incubation, the cells were washed 3 times with PBS which contained $CaCl^2$ and $MgCl_2$ to remove any free biotin. The cells were washed with 0.05 M Glycine-PBS buffer to block any free amines. Then, the cells were washed three times with PBS to wash way blocking buffer. 1 ml of DMEM-FCS cell culture medium was added and the samples were incubated at 37^{o}C for different time intervals. As positive control one well was biotinylated, immediately scrapped off. As negative control one well was not biotinylated. At the end of incubation time the cell and supernatants were collected, analyzed by Western blot as previously described.

2.2.3.5 Affinity Purification of biotinylated proteins

Streptavidin is a biotin binding protein that is isolated from *Streptomyces avidini* and has molecular mass of 60 kDa. The interaction between streptavidin and biotin is characterized by the formation of high affinity interaction. This high affinity ensures that once formed, it will not be disturbed by changes in pH, or processes such as multiple washings. Immobilized streptavidin on agarose beads can be used to purify biotinylated cell surface receptors.
The cells and supernatants from biotinylated samples were collected by transferring the cell culture into fresh tube and centrifuging it at 4000 x g for 10 minutes to pellet down the dead cells. Then free-cell culture supernatant was transferred in to a new tube. The samples were lysed in 200 µl PIRA buffer utilizing the protein extraction protocol (3.2.1.1) and transferred into fresh tube. In both tubes 60 µl of streptavidin Agarose beads were added and incubated at 4^{o}C on a rotator overnight. Next beads were centrifuged at 800 x g for 1 minute, and

supernatant was discarded. 500 µl of PBS was added to wash the beads, and centrifuged at 800 x g for 1 minute, washing step repeated twice. The beads were resuspened in 60 µl 2.5x Laemli sample buffer containing 75 mM β-mercaptoethanol and boiled at 98^{o}C for 10 minutes. The eluted proteins were separated via 11% tricine gel electrophoresis and detected by Western blot.

2.2.3.6 Quantification of biotinylation

To determine the efficiency and quantity of biotinylation the cells were stained using Cy^{TM}3-conjugated streptavidin (Jackson ImmunoResearch) and analyzed by flow cytometry. From a 6 well plate one well was biotinylated with 0.5 mg/ml and another well with 1 mg/ml to determine which concentration of biotin is required for optimal biotinylation. Unbiotinylated cells served as control was. The cells were washed two times with PBS, followed by three washing with PBS which contains $CaCl_2$ (0.0901 mM) and $MgCl_2$ (0.493 mM) (Gibco). The cells were incubated for 1 hour on ice by gentle shaking in presence and absence PBS-Biotin (EZ-link -Sulfo-NHS-LC-Biotin from Pierce) solution. After incubation, the cells were washed 3 times with PBS which contained $CaCl_2$ and $MgCl_2$ to remove any free biotin. The cells were washed with 0.05 M Glycine-PBS buffer to block any free amines. Then, the cells were washed three times with PBS to wash way blocking buffer. Immediately after washing the cells were removed by trpsin-EDTA treatment. 10 µl of biotinylated and unbiotinylated samples were transferred into a FACS tube having 100 µl FACS buffer which contained Streptavidin-Cy3 washing (1:40). As negative control no Streptavidin-Cy3 staining was done. To determine apoptotic cells, the samples were stained in 200 µl FACS buffer containing PI diluted 1:200 from a stock concentration of 1 mg/ml. The staining was verified by flow cytometric analysis.

2.2.4 Cell biological methods

General cell culture work was performed under sterile conditions under a laminar flow hood using disposable plastic ware.

2.2.4.1 Culturing

Cell lines (Table 6) were placed in polystyrene culture flasks 25 cm2 (T25) and 75 cm2 (T75) (Falcon) provided with 0.2 µm hydrophobic vent caps and maintained in a humidified 5% CO2 atmosphere in an incubator (Hera Cell 240) at 37°C. Glass wares were sterilized by heating at 180°C for 8 hours. For sub-culturing adherent cells were cultured until sub-confluence of 70-90%. Medium was removed and cells were washed with pre-warm PBS. The cells were incubated with 1.5 ml trypsin-EDTA for 3-5 minutes at 37°C. Subsequently, the cells were transferred to a tube containing a double volume of culture medium to block the trysin. Cells were pelleted harvested by centrifugation at 250 x g for 3 minutes at room temperature. The supernatant was removed and cells were washed with cell culture medium. The cells were diluted approximately 1:10 with fresh medium and seeded into new culture flask.

2.2.4.2 Freezing and thawing of cells

Adherent cells were removed from the flask by 0.05% trypsin-EDTA treatment. 5 x10^6 cells were transferred to a tube, pelleted at 250 x g for 3 minutes and supernatant was completely removed. The cells were then resuspended in 1.8 ml serum-free freezing medium. Immediately the cells were transferred into prelabelled freezing tubes and stored at -80^{o}C for one month. Thereafter, cells were transferred into liquid nitrogen for long-time storage.

Cryopreserved cells were thawn rapidly. Using a Pasteur pipette a few drops of pre-warmed cell culture medium were added into the tube and pipetted up and down for the cells to thaw and mix. Using the same pipette the thawed cells were immediately transferred into a tube containing 8 ml of warm cell culture media. Then the cells were centrifuged at 250 x g for 3 minutes at room temperature. After removal of the medium the cells were resuspended in 1 ml of media culture and transferred into a flask containing 10 ml cell culture medium. The flask was then placed into 37^{0}C incubator.

2.2.4.3 Determination of cell number

A cell suspension diluted 1: 100 with 1x PBS was used for determination of cell number. With a cover slip in place 10 µl of diluted cells were mounted in a Neubauer chamber (Assistant, Germany).

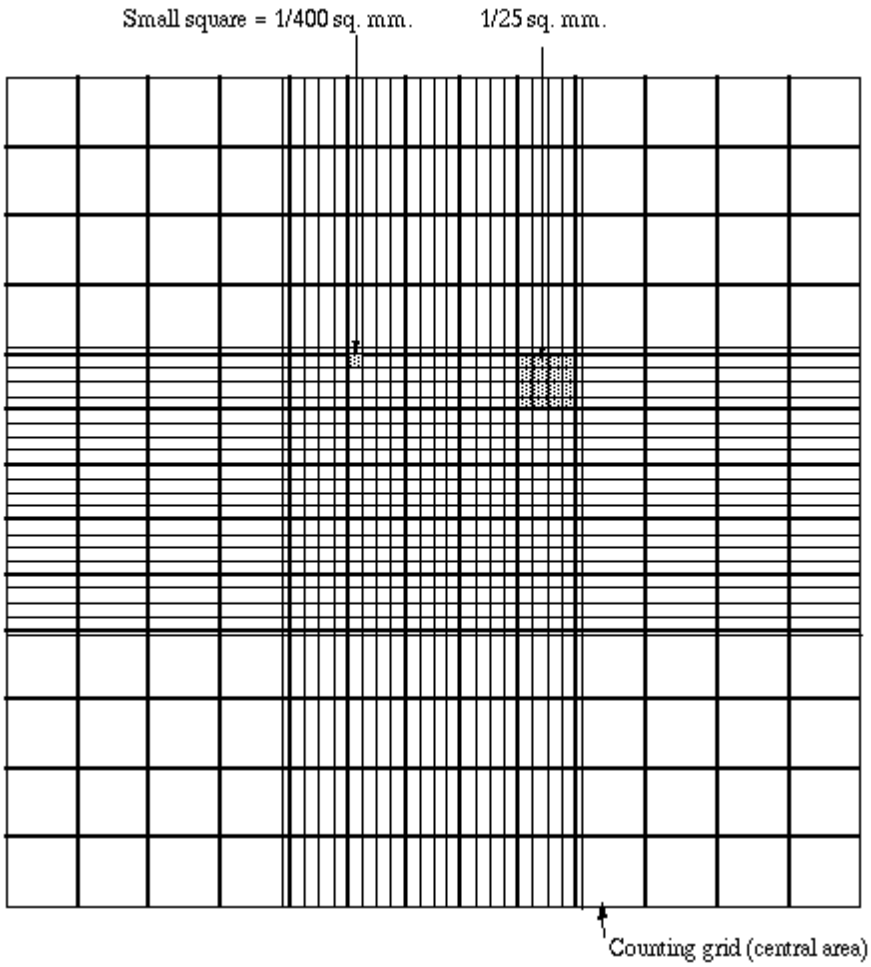


Figure 10: A drawing showing the squares on the Neubauer chamber.
This is obtained from www. Biochem.northwestern.edu/protocols.

The cell number could be calculated after counting cells in all four quadrants of the chamber, each containing sixteen smaller squares, with the following equation:
Number of cells/ml = average count per square x the dilution factor x 10^4.

2.2.5 Molecular biological methods

2.2.5.1 Isolation of total RNA

Total RNA from cells were isolated using QIAGEN RNeasy mini kit. To avoid RNAse contamination, disposable plastic ware and RNAse-free water was used. The cells were trypnized and a fraction of 2 $X10^6$ cells were taken for RNA isolation. The cells were transferred into an Eppendorf tube centrifuged at 250 x g for 3 minutes at room temperature, then supernatant was aspirated. The centrifugation was repeated to completely remove the remaining medium. The cell pellet was rescuspended in 350 µl of RT Buffer which contained β-mercaptoethanol at a ratio of 1:100 for lyzing by pipetting up and down for at least 15-20 times. The cell lysates were stored at -20^oC or directly RNA isolation was performed. The frozen cell lysates were thawn on ice and passed at least 20 times through a blunt 20-gauge needle of 0.9 mm diameter fitted to an RNase free syringe. The rest of the protocol was done according to manufactures instructions. DNAse treatment was done was then performed by on-column DNase digestion using the same kit. The RNA was eluted using 50 µl RNase-free water. Usually the concentration of RNA is determined by measuring the absorbance at 260nm (A_{260}) in a spectrophotometer. RNA has its absorbance at 260 nm and the ratio of absorbance at 260 and 280 nm is used to assess the RNA purity. Pure RNA has an A260/280 ratio of 1.8-2.1. For determination of RNA concentration an absorbance of 1 unit at 260 nm is equivalent to about 40 µg/ml (A260 = 1 = 40 µg/ml). This is valid only when the sample is in water. The concentration is calculated using the below formulae:

Concentration of RNA sample= 40 x A260 (OD) x dilution factor.

However, in our studies RNA concentration was determined by measuring the absorbance at 260 nm using Label Guard TM Microliter Cell (Implen, Germany) in a biophotometer (Eppendorf, Germany). The instrument uses two lids one having 1 mm (factor 10) and another with 0.2 mm (factor 50) and this offer an automatic dilution factor of 1/10 and 1/50. 3 µl of undiluted sample was transferred directly on to the measuring device, and then followed by placing the one of the lid. The measurement is calculated automatically in 10-15 seconds using above formulae.

2.2.5.2 Reverse transcription

1µg total RNA was reverse transcribed using QIAGEN-QuantiTect Reverse Transcription. DNA contamination was first cleansed using genomic DNA wipeout buffer supplied with the kit. The rest of the protocol was done according to manufactures instructions.

2.2.5.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR), a procedure for rapid in vitro enzymatic amplification of a specific segment of DNA, was used for the amplification of cDNA encoding CEACAM1.The template cDNA was dilute 1:5 with RNase-free water. For amplification of cDNA, 5 µl of diluted of reverse transcription product was added to 25 µl master mix (Table 14) with primers as described below. Vector pcDNA 3.1 containing CEACAM1 was used as positive control whereas water was used as negative control Glyceraldehyde-3-phosphate (GAPDH) served as a housekeeping gene to ensure equivalent DNA loading. The reaction conditions for CEACAM1 and GAPDH are shown in table below.

Components	Volume-1x
Template	5 µl
dNTP´s	0.6 µl
10x PCR Buffer	3 µl
Forward Primers	3 µl
Reverse Primers	3 µl
Taq DNA polymerase	0.2 µl
Total volume (per probe)	30 µl

Table 14: Composition of PCR reaction.

Specific primers annealing to CEACAM1 used for PCR reaction were purchased from MWG Operon (Germany) while the dNTP´s, 10x buffer and the Taq polymerase were purchased from GeneCraft. The primers for CEACAM1 and GAPDH were designated as show in Table below.

Primer Name	Primer sequences
CEACAM1 all	
Sence	AAC AGC GGT CGA GAG ACA AT
Antisense	CTC CAC AGG GTT GGA GTT GT
GAPDH	
Sense	TGA TGA CAT CAA GAA GGT GG
Antisense	TTT CTT ACT CCT TGG AGG CC

Table 15: Name and sequences of the primers used in this study.

For amplification of CEACAM1 and GAPDH cDNA 35 cycles and 29 cycles were used respectively.

Step	Temperature [°C]	Time
Initial denaturation	95	3 Minutes
CEACAM1: 35 Cycles GAPDH: 29 Cycles		
Denaturation	95	30 Seconds
Primer annealing	60	20 Seconds
Elongation	72	30 Seconds
Extension	72	8 Minutes
Cooling	4	Forever

Table 16: PCR conditions for CEACAM1 amplification

The reactions were performed in PCR plate (Biozym), using a PCR iCycler (BioRad). The GAPDH was used as control reaction. The PCR products were analyzed on a 2% agarose gel and visualized by SYBR Safe DNA gel staining (Invitrogen)

2.2.5.4 Agarose gel electrophoresis

The PCR product was electrophoretically separated on 2% (w/v) agarose gel prepared in TAE buffer containing 50X SYBR Safe DNA gel staining. 1x TAE buffer was used as running buffer. 10 µl samples were mixed with 2 µl 6x loading buffer, and then placed 11 µl of it onto the pockets gel. 5 µl of DNA molecular marker (Fermentas) was loaded into one pocket to be used for sample size evaluation. The DNA was visualized on an UV transilluminator at 302 nm and documented by a gel documentation instrument (Intas Gel Imager). For equal loading of CEACAM1 PCR product bands were judged in comparison with the GAPDH control gel.

3. The aims of the study

Previous studies have reported the presence of soluble CEA in human urine. However, the presence of CEACAM1 was neglected (Draberova et al., 2000), until most recently our group reported the presence of soluble CEACAM1 in urine of patients suffering bladder cancer (Tilki et al., 2009). However, the soluble CEACAM1 found in urine was not sufficiently investigated so far. Therefore, the tasks of this doctoral thesis were to identify the cellular origin and glycosylation stutus of CEACAM1 found in urine. In addition, to investigate which isoform or domain structure of CEACAM1 is present in urine. Furthermore, we wanted to investigate the mechanism leading to generation and secretion of this CEACAM1 form.

4. Results

4.1 Characterization of soluble CEACAM1 in urine

4.1.1 Determination of CEACAM1 and CEACAM5 in human urine

We first quatified the levels of CEACAM1 and CEA (CEACAM5) concentrations in healthy individuals using sandwich ELISA we recently developed. Our results showed concentration of 7 ng/ml ± 5 ng/ml (n=12) for CEA (Figure 11 left panel). This result agreed very well with the CEA concentration known from the literature (Guinan et al., 1974). Surprisingly, CEACAM1 concentration was much higher having 200 ng/ml ± 110 ng/ml (n=16) (Figure 11 right panel).

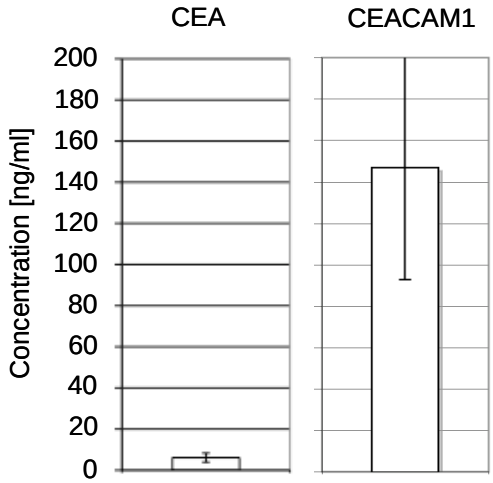


Figure 11: Determination of the CEA and CEACAM1 level in human urine. The concentration of CEA and CEACAM1 in urine was determined by sandwich ELISA. A polyclonal CEA antibody was coated on the wells of an ELISA plate. The captured molecules were detected using monocloconal antibodies specific for CEA and CEACAM1 respectively as described in the method section. The absorbance was read at 450 nm.

4.1.2 Urine CEACAM1 variant at 72 kDa

Depending on the cell type the molecular weight of CEACAM1 can be at 120-160 kDa due to differences in glycosylation (Kannicht et al., 1999; Lucka et al., 2005). For example, CEACAM1 on PMNs cells have a molecular weight of 160 kDa (Lucka et al., 2005) whereas epithelial and endothelial cells have 120 kDa and 140 kDa respectively (Bamberger et al., 1998; Prall et al., 1996). However, due to the protein marker we used in this study sometimes the molecular weight was higher or lower. Therefore, we decided to use 120 kDa to represent full length CEACAM1.

In order to further verify, the presence of CEACAM1 in urine and to determine its molecular weight immunoprecipitation was performed. The immunoprecipitated samples were analyzed by Western blot and detected using an anti-CEACAM1 monospecific antibody. As positive control HeLa-CEACAM1-4L cell lysates was included. In parallel Western blot analyses were performed for detection of CEA from human urine and from HeLa-CEA cell lysates as positive control. Then blots were detected using anti-CEA monoclonal antibody. Western blots showed the expected molecular weight of CEA (180 kDa) in HeLa-CEA cell lysates as well as in urine. In addition, 120 kDa of the known molecular size of CEACAM1 was detected in the HeLa-CEACAM1 probe. However, in urine a band below 75 kDa was detected (Figure 12). Calculation utilizing protein standard revealed a molecular weight of 72 kDa for the CEACAM1 found in urine. This band was most recently reported (Tilki et al., 2009) by our group and it was stated to represent a soluble CEACAM1 variant. However, nothing about the biochemical set up and its origin was shown yet.

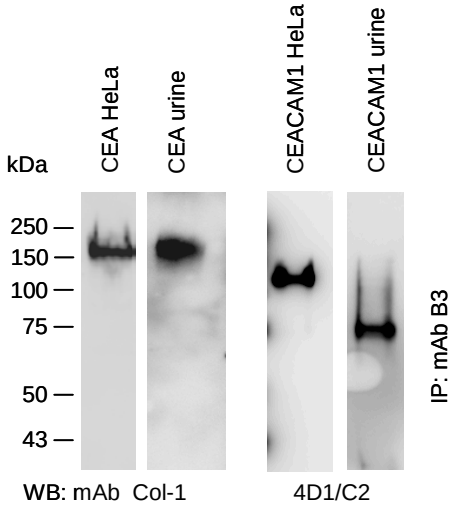


Figure 12: Detection of CEACAM1 and CEA expressed by transfected HeLa cell in comparison with those found in urine.
Immunoblot analysis was performed as described in method part. The experiment shown is representative of three different experiments.

4.1.3 Soluble and membrane bound CEACAM1 in human urine samples

To determine whether CEACAM1 found in urine represents the soluble or the membrane-anchored form, TX-114 based phase separation assay was performed. The separated fractions were subsequently analyzed by sandwich ELISA to quantify amount of CEACAM1 in each fraction (Figure 13A). The concentration of CEACAM1 in this cohort of healthy donors was 100 ng/ml ± 10 ng/ml (n=4) in the control urine prior to the separation into a hydrophilic and a hydrophobic fractions. After the phase separating centrifugation the hydrophilic fraction contained 70 ng/ml ± 5 ng/ml (n=4) whereas the hydrophobic fraction contained only 10 ng/ml ± 5 ng/ml (n=4).

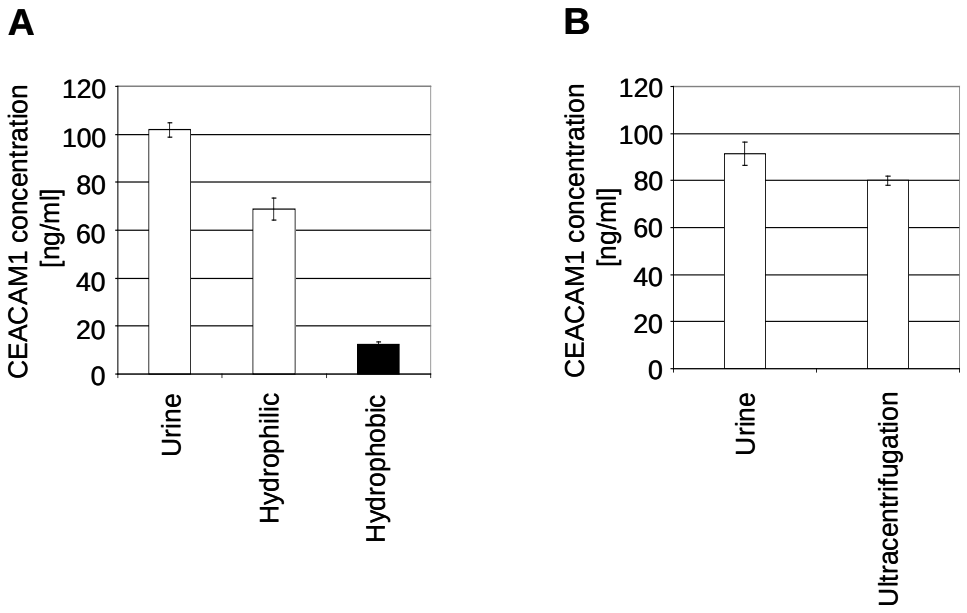


Figure 13: Characterization of soluble CEACAM1 found in urine.
A) Urine sample was collected and subjected to phase separation assay using Triton X-114. Subsequently, each fraction obtained was analyzed by sandwich ELISA for determination of the CEACAM1 concentration. B) Urine sample was collected and subjected to ultracentrifugation for two hours and the upper layer transferred for further analysis. Then the sample together with a control of native urine was analyzed for the determination of CEACAM1 concentration using sandwich ELISA. The experiments shown are representative data of three different experiments.

To confirm the results gained with phase separation assay, ultracentrifugation of the urine samples followed by sandwich ELISA was performed. For that urine samples were taken and divided into two portions. One was kept untreated as control urine while the other portion was subjected to the ultracentrifugation step. After ultracentrifugation the upper layer was carefully transferred into a new tube without disturbing the pellet. Then both the control urine and the ultracentrifugated portion were analyzed by sandwich ELISA to determine the

concentration of CEACAM1. As shown (Figure 13B) 80 ng/ml ± 5 ng/ml (n=4) were water soluble which equal to 88.9% whereas 10 ng/ml ± 2 ng/ml (n=4) were membrane bound. Thus leads to conclude that the main CEACAM1 fraction in urine is soluble and not membrane bound.

4.1.4 Reduced molecular weight of urine CEACAM1 and its glycosylation.

The extracellular domains of CEACAM1 are heavily glycosylated, generally more than half of the molecular weight of CEACAM1 consists of carbohydrate (Lucka et al., 2005). Thus, it was tempting to hypothesize that the molecular weight differences are caused by differences in gylcosylation. To analyze this idea we performed deglycosylation assays. A control assay of deglycosylated human CEACAM1-Fc construct was utilized to first determine the exact molecular weight of a deglycosylated CEACAM1. As shown in (Figure 14A) the size of the undeglycosylated human CEACAM1-Fc was approximately 120 kDa whereas following complete deglycosylation the size reduced to approximately 75 kDa. Although the CEACAM1 in human urine was only a little bit smaller compared with the deglycosylated CEACAM1-4, it could have been the answer. But deglycosylation of CEACAM1 isolated from urine revealed a band of molecular 40 kDa. (Figure 14A right panel). Thus CEACAM1 in urine is still highly glycosylated.

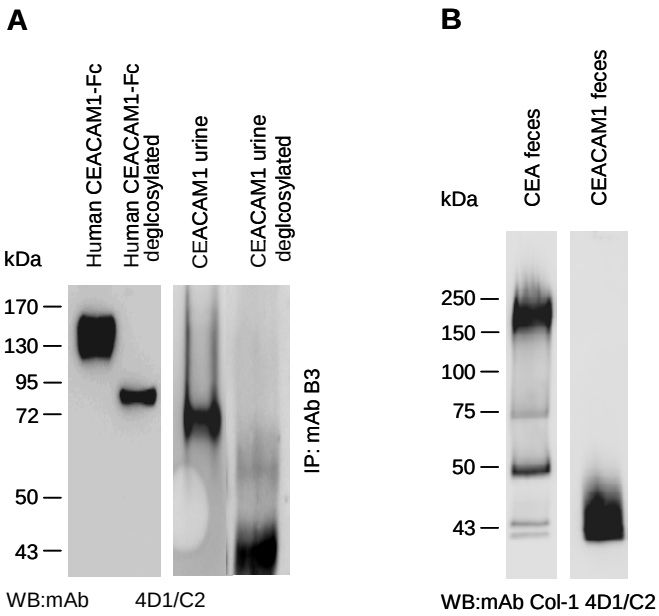


Figure 14: Deglycosylation studies of CEACAM1.
A) Deglycosylation assay was performed as described in the method part. B) Western blot analysis of CEA and CEACAM1 from feces for comparison with degylcosylation assay. These are representative data of three separate experiments.

In addition the concentration of CEA in feces was determined using sandwich ELISA and found to be approximately 1 mg/ml (data not shown) which corresponded well with what was previously reported (Elias et al., 1974). Feces samples were analyzed by Western blot for CEA and CEACAM1 (Figure 14B). Detection of CEA in feces showed a broad band at approximately 180 kDa and additional faint bands at 75 kDa, 50 kDa, 45 kDa, and at 40 kDa. On the other hand, detection of CEACAM1 showed only one broad band at 40 kDa which corresponds to that of deglycosylated CEACAM1 in urine.

4.1.5 Urine CEACAM1 without N-Domain.

To determine whether the decrease in molecular weight of CEACAM1 in urine may be caused by changes in the number of CEACAM1 domains epitope mapping using sandwich ELISA was performed. This was done by applying different monoclonal antibodies binding to distinct epitopes at different CEACAM1 domains. The detecting antibodies used were specifically binding to the various domains as follows: 18/20, R+D (N-domain), B3 (A1 domain), C5-1X (B domain) 4D1/C2 (the linker between B-A2 domains), 8G5 (A2 domain) and anti-CEACAM1 cyto (cytoplasmic region). The signal intensity of Hela-CEACAM1-4L (control) in total cell lysates was determined by optical density (OD) and taken as 100%. Then the amount of CEACAM1 detected in the urine sample was calculated as percentage of the positive control detected by the same antibody. As shown (Figure 15A) CEACAM1 was not detected by both antibodies that bind to the N-domain. Also the monoclonal antibody specific for the A2 domain and the cytoplasmic region of CEACAM1 were negative. In contrast, the antibody that binds to the A1-domain and another that binds the linker between B and A2- domains were strongly positive.

To confirm that urine CEACAM1 does not have N-domain, a part of the A2 and the entire cytoplasmic region, urine samples were subjected to ultracentrifugation with subsequent epitope mapping using the different antibodies as described above. Detection of CEACAM1 using monoclonal antibody that binds to A1B domains showed that hydrophilic fraction contained 90% ± 5% CEACAM1 whereas the hydrophobic contained only 10% ± 5% (Figure 15B). The optical density of each fraction was calculated as percentage of the 100% total urine. CEACAM1 was not detected by monoclonal antibodies that binds to the N-domain A2 domain and the cytoplasmic region (data not shown). The results indicated that CEACAM1 in urine does not have the N-domain, part of A2-domain and the cytoplasmic region. Thus, in this study the 120 kDa CEACAM1 was referred to as full length while the 72 kDa CEACAM1 variant was termed as truncated CEACAM1.

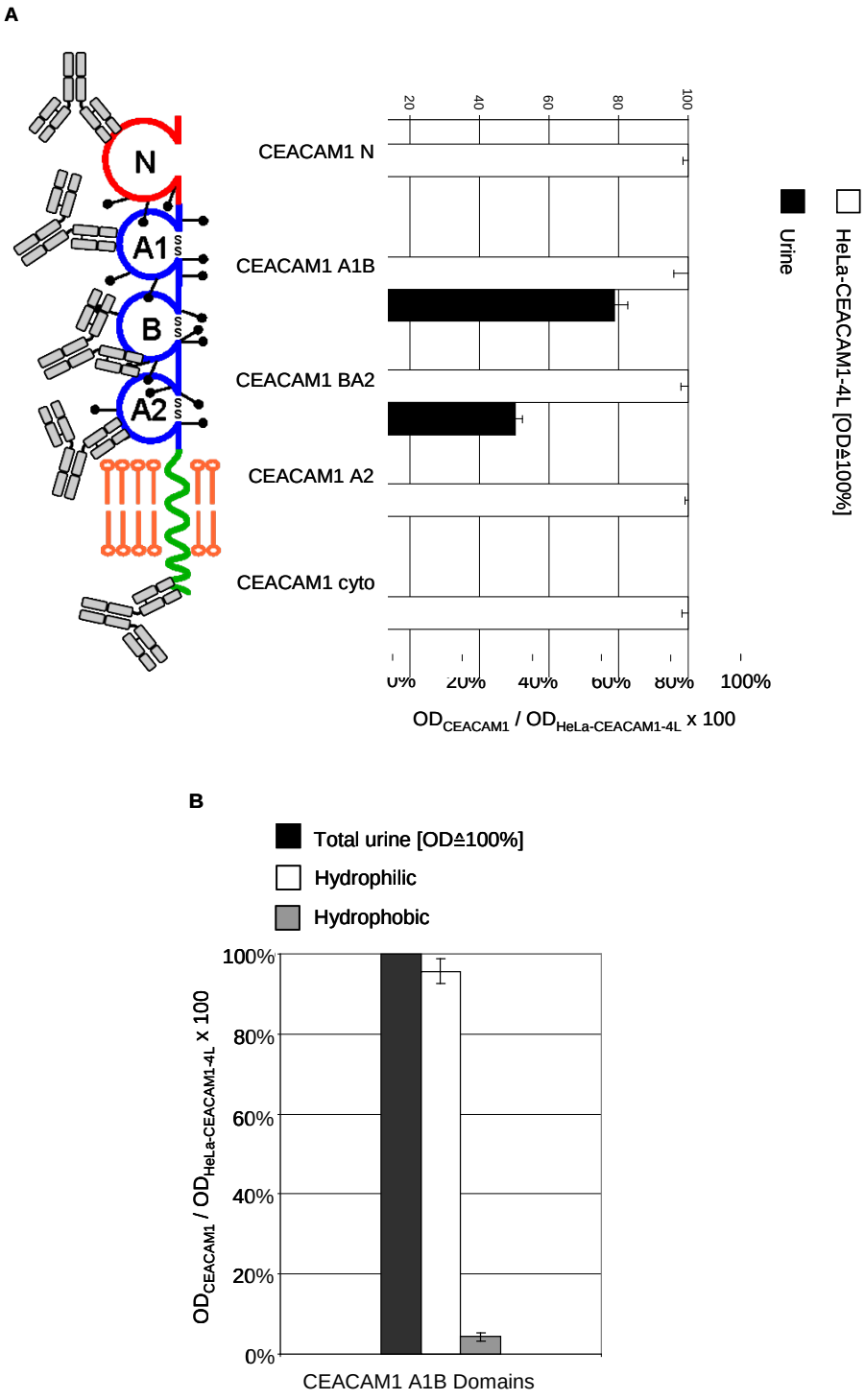


Figure 15: Epitope mapping of CEACAM1 in urine.
A) The plate was coated with polyclonal anti-CEA antibody and the captured CEACAM1 molecules were detected using different monoclonal antibodies specific for distinct epitope as described above. The amount of CEACAM1 bound was measured in absorbance at 450 nm. It was measured in triplicates. HeLa-CEACAM1 cell lysates was used as positive control for the monoclonal antibody. B) Urine sample was subjected to ultracentrifugation and domains recognized in each fraction were quantified by sandwish ELISA. A control of total urine was included. Then the optical densities obtained were calculated in percentages in relation to control. These are representative data of three separate experiments.

4.2 Characterization of CEACAM1 in human granulocytes

4.2.1 Expression of full length but not truncated CEACAM1 in human granulocytes

It is well known that human granulocytes (PMN) express high amounts of CEACAM1 with a molecular weight of 160 kDa molecular weight (Drzeniek et al., 1991). In order to identify whether granulocytes could be the likely source of the 72 kDa soluble CEACAM1 found in urine Western blot analysis were preformed. CEACAM1 was detected using two distinct mouse monoclonal antibodies 18/20 (binding to the N-domain) and 4D1/C2 (binding to the liker between A1 and B domains). As shown (Figure 16) both antibodies detected only the described full length CEACAM1 with a molecular weight of 160 kDa but not the truncated CEACAM1. These results confirm the expression of CEACAM1 on granulocytes and show that no truncated CEACAM1 was identified.

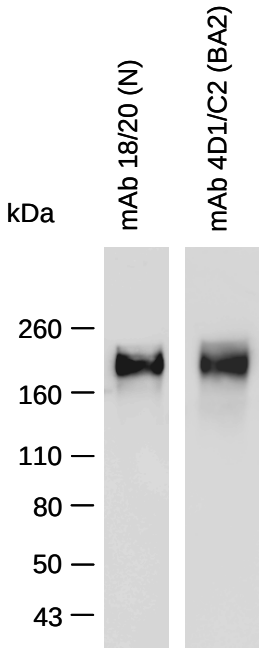


Figure 16: Identification of CEACAM1 expressed in human granulocytes.
Western blot analysis of CEACAM1 from whole lysates of human granulocytes was done as described in the method part using antibodies directed against CEACAM1. The results shown are representative of three independent experiments.

4.2.2 No detection of truncated CEACAM1 in human granulocytes under spontaneous or ligand induced apoptosis

Normally, granulocytes are short-lived cells with a half-life of only 6-20 hours in the circulation, after which they undergo constitutive (spontaneous) apoptosis. Previous studies reported that healthy donors produce and destroy 180 x 107 granulocytes per day (Haslett et al., 1991; ATHENS et al., 1961). Thus, we wanted to analyze whether truncated CEACAM1

may appear in granulocytes due to spontaneous apoptosis. PMNs cells freshly isolated were either kept or cultured for 24 hours as described in the method part. The whole cell lysates of the freshly isolated control, PMNs cells after 24 hours and their cell culture supernatant were collected and analyzed by Western blot. The results showed 160 kDa bands representing full length CEACAM1 in both fresh isolated and apoptotic granulocytes (Figure 17A). A minor band of 160 kDa was detected in the cell culture supernatant indicating minimal release of full length CEACAM1 due to apoptosis. If this released form appeared due to shedding or due to microvesicles (MV) was not analyzed in this work. Flow cytometric analysis after staining the cells with Annexin V-FITC and propidium iodide confirmed spontaneous apoptosis (data not shown). There was no 72 kDa band detected in any of the sample indicating that truncated CEACAM1 is not generated due to spontaneous apoptosis of granulocytes.

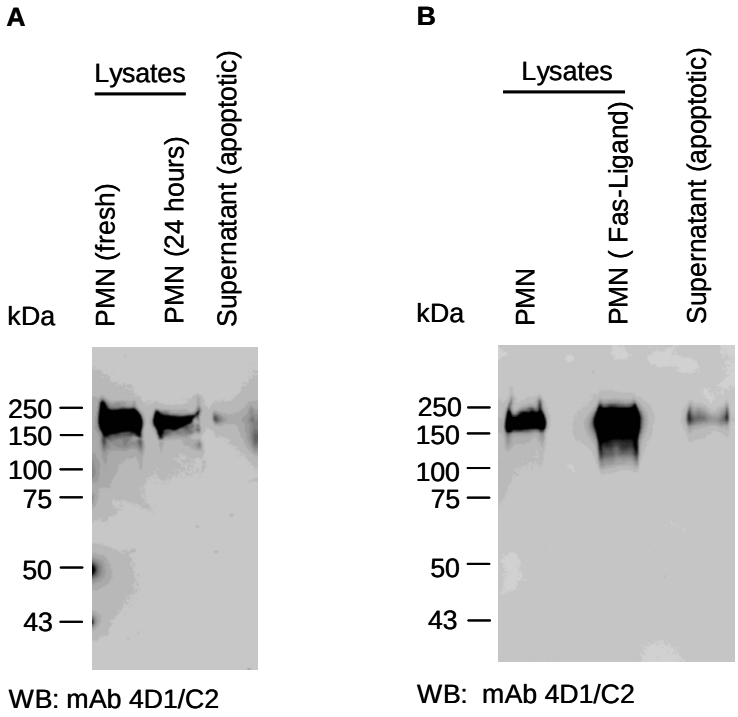


Figure 17: Effect of CEACAM1 found in granulocytes due to spontaneous and Fas-ligand induced apoptosis.
A) Western blot analysis of cell lysates and cell culture supernatant obtained before and after spontaneous apoptosis. B) Western blot analysis of cell lysates and cell culture supernatant before and after Fas-Ligand induced apoptosis. All blots were developed using monospecific anti-CEACAM1 antibody. These are representative data of three separate experiments.

The expression of Fas receptors on the surface of granulocytes as reported (Liles et al., 1996) implicated the possibility the granulocytes initiate a death pathway at the cell surface. To test whether the truncated CEACAM1 may appear due to Fas ligand induced apoptosis

freshly isolated PMN were cultured in the presence and absence of soluble Fas ligand. The whole cell lysates of a control of freshly isolated granulocytes, FasL-induced sample, and its cell culture supernatant were analyzed for CEACAM1 expression by Western blot. The results showed 160 kDa bands representing the full length CEACAM1 in both freshly isolated as well as apoptotic granulocytes (Figure 17B). A weak band with 160 kDa was also detected in the cell culture supernatant indicating minor release of full length CEACAM1. Flow cytometric analysis of Annexin V-FITC/propidium iodide staining confirmed FasL-induced apoptosis (data not shown). There was no 72 kDa band detectable in any of the samples indicating that in granulocytes truncated CEACAM1 is also not generated by ligand-induced apoptosis.

4.2.3 No association of truncated CEACAM1 to PMN ingestion by macrophages

Usually apoptotic or senescent granulocytes are cleared using phagocytosis by macrophages. To determine whether, the truncated CEACAM1 might appear due to ingestion of granulocytes by macrophages freshly isolated PMNs were co-cultured with macrophages as described in the method part. Then all samples fractions were analyzed by Western blot analysis. As shown (Figure 18) a 160 kDa band of full length CEACAM1 could be detected in lane 1, 2 and 5 which originated from freshly isolated PMNs, cultured PMNs, and suspension cells of macrophages in presence of PMN respectively. Very faint bands of full length CEACAM1 could be detected in cell culture supernatants of PMNs confirming a minimal release of full length CEACAM1 after spontaneous apoptosis and in the adherent cells of macrophages in presence of PMN. This may be due to the already attached PMN cell on the macrophages. However, no band was detected in the supernatant of the macrophages in presence of PMN cells, adherent macrophages, also in macrophages in suspension and in supernatants of macrophages. Furthermore, in none of the samples the 72 kDa band of the truncated CEACAM1 was found. Lack of any detectable CEACAM1 in the cell culture supernatant of the macrophages in presence of PMNs suggest that CEACAM1 on granulocytes was completely digested by macrophages. These results demonstrated clearly that truncated CEACAM1 found in the urine does not result from ingestion of granulocytes by macrophages.

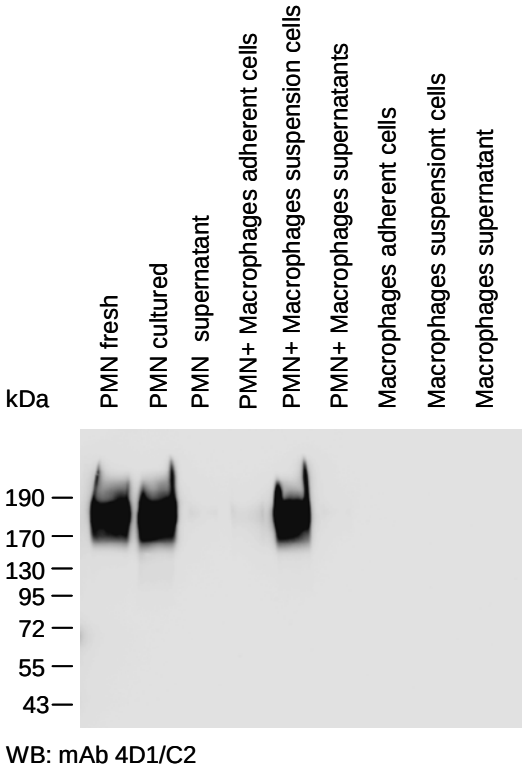


Figure 18: CEACAM1 expression in granulocytes co-incubated with macrophages. Western blot analysis of whole cell lysates and supernatant from granulocytes and macrophages not incubated and co-incubated with macrophages was performed applying CEACAM1 monospecific antibody. The rest was done as described in the method part. The findings shown are representative of three independent different experiments.

4.3 Characterization of CEACAM1 in epithelial cells

4.3.1 Epithelial cells as a potential source of truncated CEACAM1

In order to further investigate the putative origin of the truncated CEACAM1 found in human urine the following epithelial cell lines known to express CEACAM1 endogenously (A549, HT29, and T102/3,) and stable transfected CEACAM1-4L cell line (HeLa-CEACAM1-4L) were cultured as described in the method part. Immunoprecipitation was performed on the whole cell extracts using monoclonal antibody 4D1/C2. Detection using the monoclonal antibody 18/20 that binds to the N-domain of CEACAM1 revealed a 120 kDa band of full length CEACAM1 in all cell types analyzed (Figure 19A). Interestingly, detection using the monoclonal antibody 4D1/C2 resulted in two bands; one at 120 kDa and another one at 72 kDa in each of the cell line (Figure 19B). These results confirmed the presence of full length CEACAM1 and identified a 72 KDa CEACAM1 in epithelial cell which was not detected by the N-Domain binding antibody 18/20. Thus, epithelial cell are very likely to be the source of truncated CEACAM1.

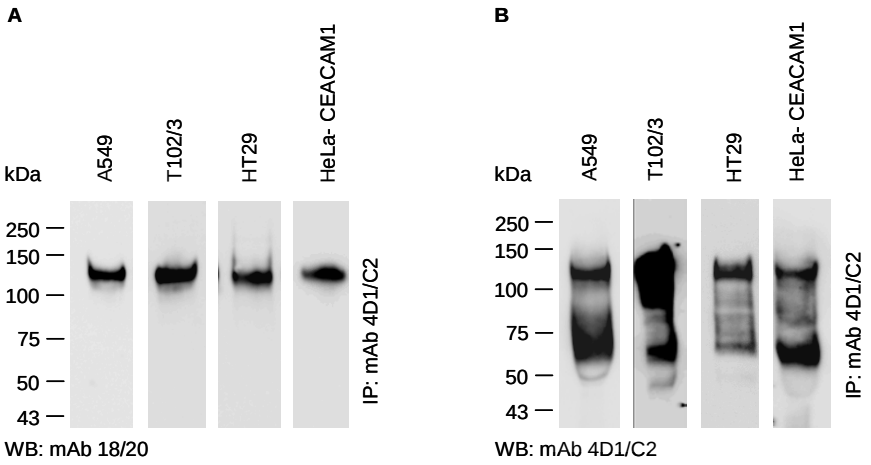


Figure 19: Expression of both full length and truncated CEACAM1 in different cells lines
Immunoprecipitation and Immunoblot of whole cell lysates from A549, T102/3, HT29 and CEACAM1 transfected HeLa cells (HeLa-CEACAM1-4L) was performed as described in the method part. Anti CEACAM1, which binds to N-domain showed full length CEACAM1 whereas antibody binding to the linker between B-A2 domains showed that truncated CEACAM1 is additionally expressed in these cell lines. These are representative data of three separate experiments.

4.3.2 The appearance of 72 kDa CEACAM1 and glycosylation

Having identified various epithelial cell lines as well as transfectants as putative source for the truncated CEACAM1, we further characterized this 72 kDa CEACAM1 form. Deglycosylation assay was performed to determine whether the decrease of CEACAM1 from

120 kDa to 72 kDa may appear due to differences in glycosylation. Immunoprecipitation experiments were carried out utilizing monoclonal antibody B3 followed by Western blot using non-glycosylated and deglycosylated samples (Figure 20). As shown in the A549 cells sample (lane 1) both 120 kDa and 72 KDa of non-deglycosylated CEACAM1 were detectable. However, after treatment with PNGase enzyme resulted in weak bands at 75 kDa and at 40 kDa representing the non-deglycosylated CEACAM1 of 120 kDa and 72 KDa respectively. In T102/3 samples (lane 2) the main band appeared at 120 kDa and additional fainter band at approximately 72. However, no band was detected for deglycosylated CEACAM1. Finally in HT29 cells both 120 kDa and 72 kDa were detected whereas following degylcosylation only a 40 kDa band was detected. These results clearly show that deglycosylation of 120 kDa and 72 kDa leads to 75 kDa and 40 kDa bands respectively. Furthermore, the decrease of 72 kDa to 40 kDa corresponds well with the deglycosylation assay of soluble CEACAM1 in urine. The 72 kDa CEACAM1 variant found in urine is still glycosylated. Thus deglycosylation of CEACAM1 resulting in a decrease of the molecular weight from 140 kDa to 75 KDa can not be counted as the source of the 72 kDa CEACAM1 variant. Interestingly, it was noted after degylcosylation the affinity to the antibody was very low thus resulting to faints band and this may suggest that sugars are of important in the binding of the antibody.

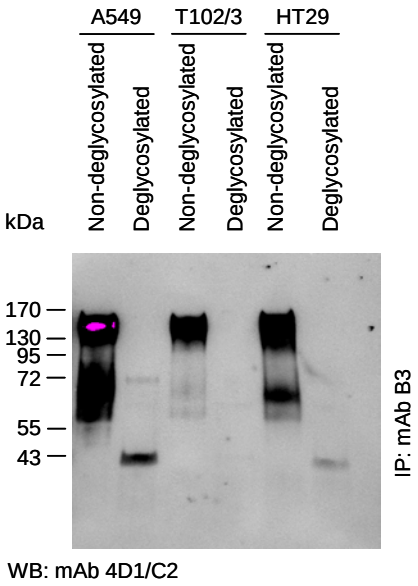


Figure 20: Characterization of non-deglycosylated and deglycosylated CEACAM1 expressed in epithelial cells.
Deglycosylation assay was performed using PNGase F enzyme treatment on immunoprecipited CEACAM1. Subsequently, the samples were analyzed by Western blot and detected by a monoclonal anti-CEACAM1 antibody. The experiment was repeated twice.

4.3.3 Truncated CEACAM1 expressed in epithelial cells lacks the N-domain

To determine whether the 72 kDa CEACAM1 variant found in epithelial cells lacks the N-domain further characterization using epitope mapping was performed. Immunoprecipitation of CEACAM1 from A549 cells with antibody specific for A1-B domains and another one specific for B-A2 domains was performed as described in the method part. Furthermore, detection using an antibody 4D1/C2 binding to the B-A2 domains recognizes both bands (Figure 21 right panel). However, detection using an antibody 18/20 binding to the N-domain recognizes only the full length at 120 kDa but not the 72 kDa (Figure 21 left panel). This experiment was also performed using T102/3 and HT29 and showed similar results (data not shown).These results clearly proofs that the 72 kDa form of CEACAM1 expressed in the epithelial cells lacks the N-domain. In line with the epitope mapping of soluble CEACAM1 found in urine it clearly indicates that the 72 kDa form is a truncated variant of CEACAM1 which does not have the N-domain, a part of A2-domain and the cytoplasmic region.

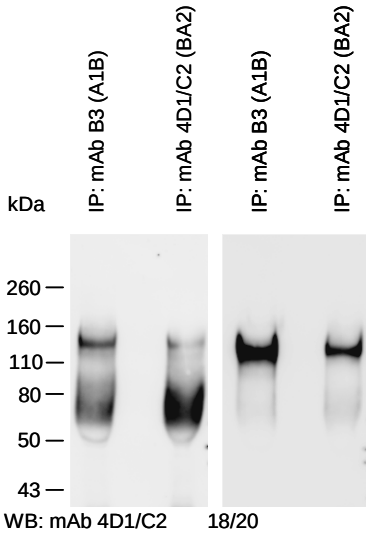


Figure 21: Epitope mapping of lower molecular CEACAM1 expressed by A549 cells. Determination of epitopes was performed by immunoprecipitation with two distinct monoclonal antibodies that binds at A1-B and B-A2 domains respectively. Then recognition of the precipitated CEACAM1 was determined by Western blot using two antibodies, one for the N-domain, one for the A1-B domains. The experiment shown is representative of three different experiments.

4.3.4 Appearance of truncated CEACAM1 and apoptosis

It was previously reported that induction of apoptosis by various agencies leads to cleavage of CEACAM1 (Nittka et al., 2008). This results in a 58 kDa and a 14 kDa fragment. Thus, it was obvious to speculate that the truncated CEACAM1 found in the urine may appear due to apoptosis as well. To analyze that idea we utilized our cell lines and induced apoptosis using actinomycin, campthecin, cycloheximide, dexamethasone and etoposide. Then we controlled the apoptosis by Annexin V-FITC/PI staining and FACS analysis. Because all apoptose

inducing agents had almost similar results in this study we only show the results of actinomycin. The results showed that 75% ± 5% cell survival for the controls whereas only 5% ± 3% survived in the case of induced cells. As shown (Figure 22 A and B) we could induce cell death significantly. Subsequent analyses of the expressed CEACAM1 revealed a full length but not truncated CEACAM1 (Figure 22 C). In the contrast to the published work of Nittka et al, neither 58 kDa nor 14 kDa CEACAM1 fragment could be detected. This experiment was also performed for other epithelial cells (data not shown) to eliminate the risk of failure. Therefore, apoptosis did not lead to the truncated CEACAM1.

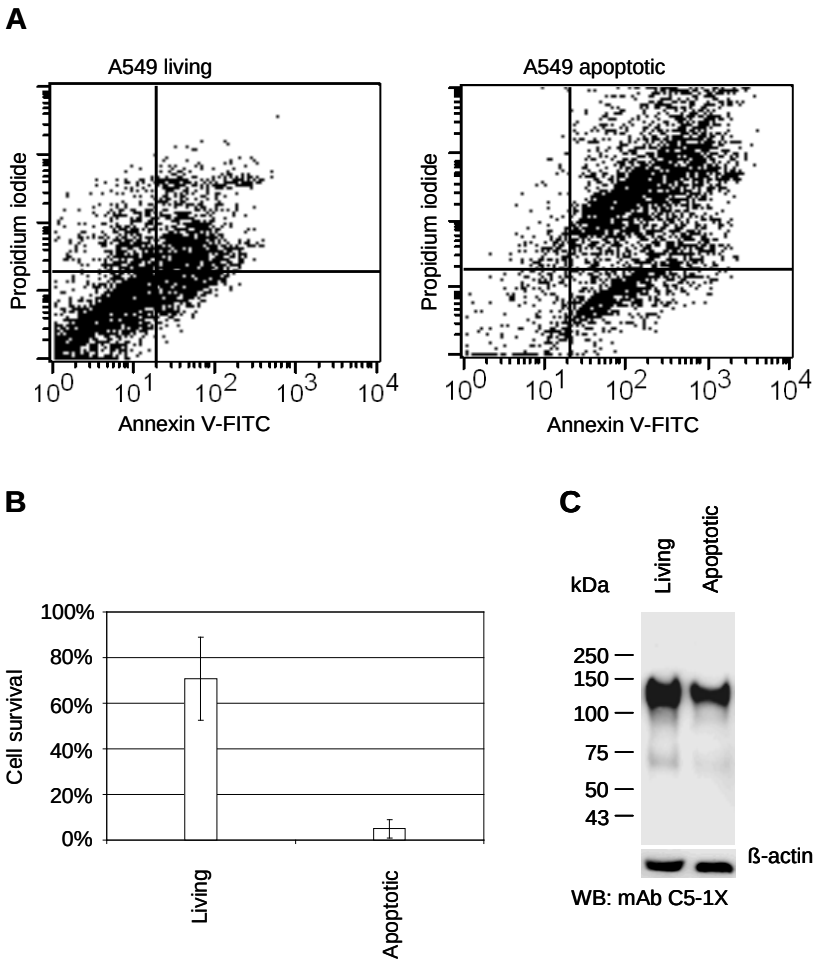


Figure 22: Induction of apoptosis in A549 cells.
A/B) Apoptosis was induced in the cells for 24 hours as described in the method part. Then, the samples were stained with Annexin V-FITC and PI and analyzed by flow cytometry. B) Shows statistics of 5% ± 3% (n=3) cell death after induced apoptosis. The results indicate percentages of cell survival which are the mean ± SD (error bars). C) Western blot of living and apoptotic A549 cells with respect to the CEACAM1 expression. The data shown are representative of three different experiments.

4.3.5 Appearance of truncated CEACAM1 and down regulation of epithelial CEACAM1

The results that A549 cells down regulated CEACAM1 when entering into the proliferative state (Singer et al., 2010) led to the idea that truncated CEACAM1 might result due to this process. They also reported that A549 cells that had just reached confluence did not express CEACAM1. However, these cells induced and therefore increased the CEACAM1 expression as soon they entered the post-confluent growth arrest state. This in mind, confluent cells which showed high CEACAM1 expression were seeded into culture vessels with low cell density and named as day 0 cells. Initially, absolute CEACAM1 expression on the surface of these cells was analyzed by quantitative flow cytometric analysis (data not shown). The data agrees with those published recently (Singer et al., 2010). The cells were constantly cultured in proliferating growth phase for one, two, three, four, and seven days, respectively. Then, the whole cell lysates were collected and analyzed by Western blot (Figure 23A). On day 0 (Figure 23A) a main band of CEACAM1 appeared at 140 kDa accompanied by a band at 72 kDa. One day later (day 1) the expression of full length CEACAM1 slightly decreased whereas truncated CEACAM1 slightly increased. The following day (day 2), the expression of both full length and truncated CEACAM1 started to decrease, and continued to decrease until very little expression of full length CEACAM1 was detectable and no truncated CEACAM1 was left. To investigate the increase of truncated CEACAM1 between day 0 and day 1 the same assay was repeated but rather for short time periods (1, 2, 4, 6, 8 and 24 hours). Results did not show any significant increase of truncated CEACAM1 (data not shown).

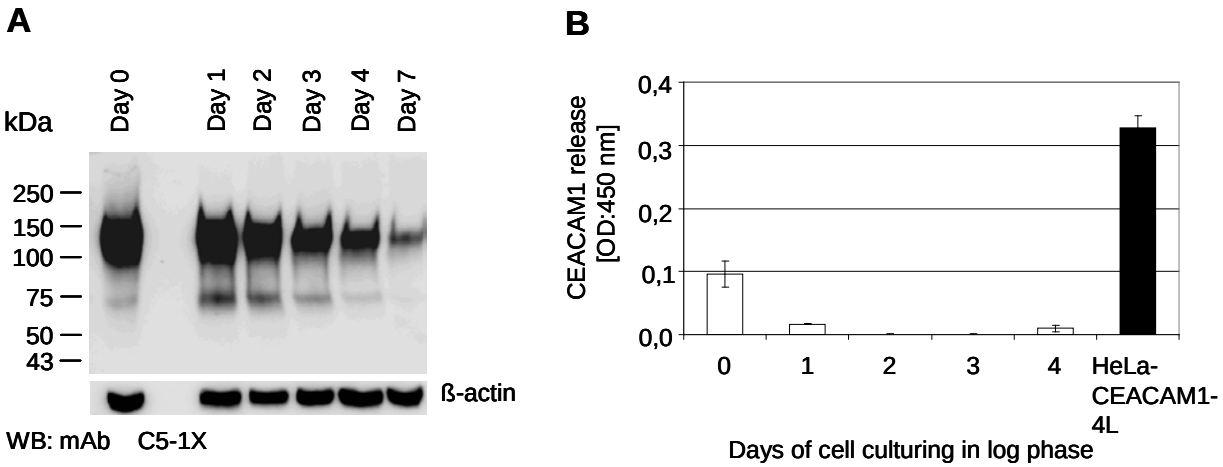


Figure 23: Reduced CEACAM1 expression in cells entering proliferation.
A) Western blot of confluent and log phase cultured A549 cell lysates was done as described in the method part. The detection of beta-actin served as loading control. B) The released CEACAM1 from confluent and log phase cultured A549 cells was quantified by sandwich ELISA as described in the method part. The values ± SD were determined from triplicates. The data shown are representative of four different experiments.

This data showed that the decrease of surface CEACAM1 does not lead to the increase of truncated form of CEACAM1. To further analyze whether the down-regulated CEACAM1 may have been shed and secreted into the cell culture supernatant, sandwich ELISA was performed (Figure 23B n=4). The results showed very little amount (0.1 ± 0.03 OD) of CEACAM1 released in cell culture supernatant on day 0, whereas nearly nothing was measurable at a later time point. According to these results the truncated CEACAM1 is not associated with reduced CEACAM1 expression in cells entering proliferation. However, the loss of both full length and truncated CEACAM1 during cell proliferation implies that the cells may need all amino acids and sugar of the degraded CEACAM1.

We next quantified CEACAM1 expression in cell growth phases of epithelial cell using quantitative flow cytometry approach was used. The cells were cultured with low density, for at least two days to be in log phase prior to analysis. To reach tight confluency, cells were cultured for additional six days after reaching confluency. The cells were stained with monoclonal antibody 18/20 and the level of CEACAM1 expression was determined in reference to calibration beads conjugated with a known amount of antigen for secondary antibody. In log phase of HT29 and T102/3 cells a significant expression of approximately 8,000 ± 1500 (n=4) molecules per cell was observed where as in case of A549 cells no CEACAM1 expression on the surface of was detected. However, a signification up-regulation with approximately 18,000 ± 4000 (n=4) molecules per cell was observed when all cell types were kept in confluency (Figure 24).

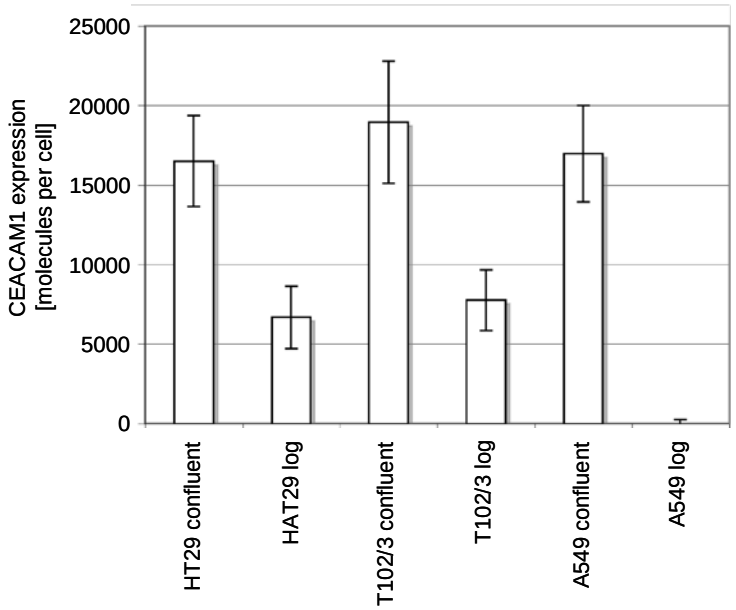


Figure 24: Characterization of the cell surface expression of CEACAM1 in different growth stages.
Epithelial cells were collected from different cell growth stages as indicated, were then analyzed by absolute quantitative flow cytometry utilizing the QuiFiKit approach as described in the method part. The values ± SD were determined from triplicates. These data are representative of four separate experiments.

4.3.6 Appearance of truncated CEACAM1 and turnover in epithelial cells

Having known that A549 cells that had reached confluence did not express CEACAM1 but the expression increased as cell entered the post-confluent contact inhibited growth stage. This led us to investigate whether, the increase of CEACAM1 expression during post-confluent phase would lead to appearance of truncated CEACAM1. Epithelial cell lines (A549, T102/3, HT29) were seeded as described in the method part. Protein extracts were collected when confluent, confluent plus one week, confluent plus two weeks, and confluent plus three weeks. Western blot analysis of A549 cells using monoclonal antibodies 18/20, C5-1X and 4D1/C2 detected little CEACAM1 expression in lysates of just confluent cells (Figure 25A). As shown (Figure 25A left panel) detection using the monoclonal antibody (18/20) specific for the N-domain of CEACAM1 detected an increase in the expression of the full length CEACAM1 in the confluent plus one week sample. This increase continued when the cells were kept in contact inhibited state for confluent plus two weeks, and confluent plus three weeks, respectively. As shown (Figure 25A middle panel) when the blots were detected using the monoclonal antibody C5-1X expression of both full length and truncated CEACAM1 increased as cells were kept in a contact inhibited state. Interestingly, a significant increase of truncated CEACAM1 could be observed by the not N-domain binding monoclonal antibody 4D1/C2 (Figure 25A right panel) when cells were grown from confluent to confluent up to three weeks. The results shown above were confirmed using T102/3 cells (Figure 25B) and HT29 cells (Figure 25C). We found that monoclonal antibody 4D1/C2 detected broad bands of truncated CEACAM1 and faint bands of full length CEACAM1 whereas, C5-1X detected broad bands of full length CEACAM1 and faint bands of truncated CEACAM1 although both monoclonal antibodies were not CEACAM1 N-domain binding. These differences may be due to the antibodies affinities or accessibility of their distinct epitopes. The presence of full length CEACAM1 but not truncated CEACAM1 when using monoclonal antibody 18/20 confirmed that the truncated CEACAM1 does not contain N-domain. These results demonstrated that both full length as well as truncated CEACAM1 appeared and significantly increased when the cells were kept in contact inhibited state. Thus, the molecular protein turnover seemed to play an important role in expression and regulation both full length and truncated CEACAM1.

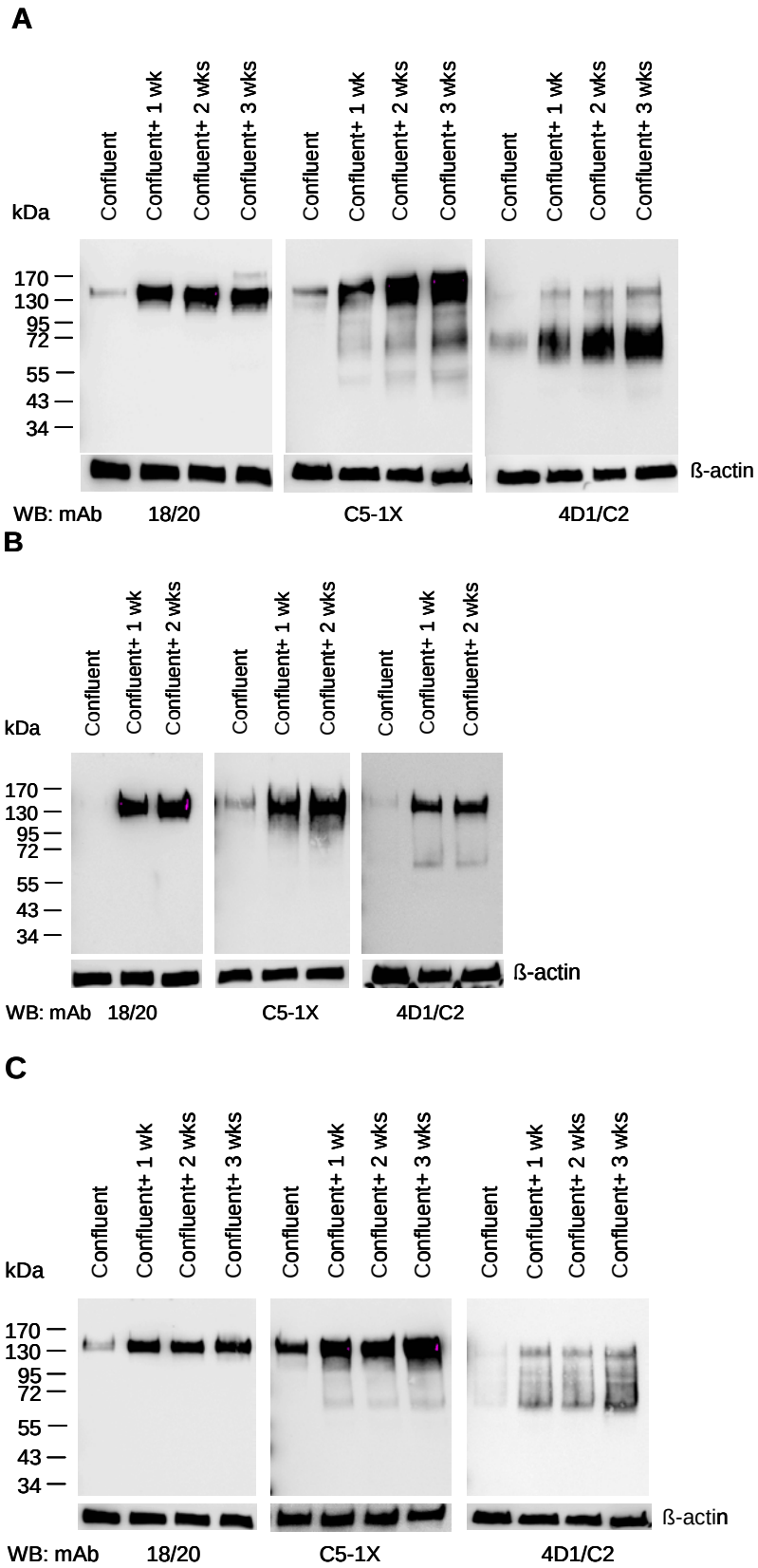


Figure 25: Up regulation of full length and truncated CEACAM1 in contact-inhibited epithelial cells.
A) A549 cells, B) T102/3 cells, C) HT29 cells were cultured for indicated time intervals. Whole cell lysates were collected after the cell growth stages as indicated above. Subsequently, samples were analyzed by Western blot and the determination of CEACAM1 was done using three distinct monoclonal anti-CEACAM1 antibodies. The detection of beta-actin served as loading control. The data shown are representative of three different experiments.

These results were expanded by absolute quantitative flow cytometric analysis of cell surface CEACAM1. The CEACAM1 expression was analyzed as cells were just confluent, confluent plus one week, confluent plus two weeks, and confluent plus three weeks (Figure 26). The results showed low expression of CEACAM1 in just confluent cells. However, the expression progressively increased attaining approximately 25,000 ± 2,200 (n=3), 41,000 ± 5,030 (n=3), and 22,000 ± 1,850 (n=3) molecules per cell in A549, T102/3 and HT29 cells, respectively.

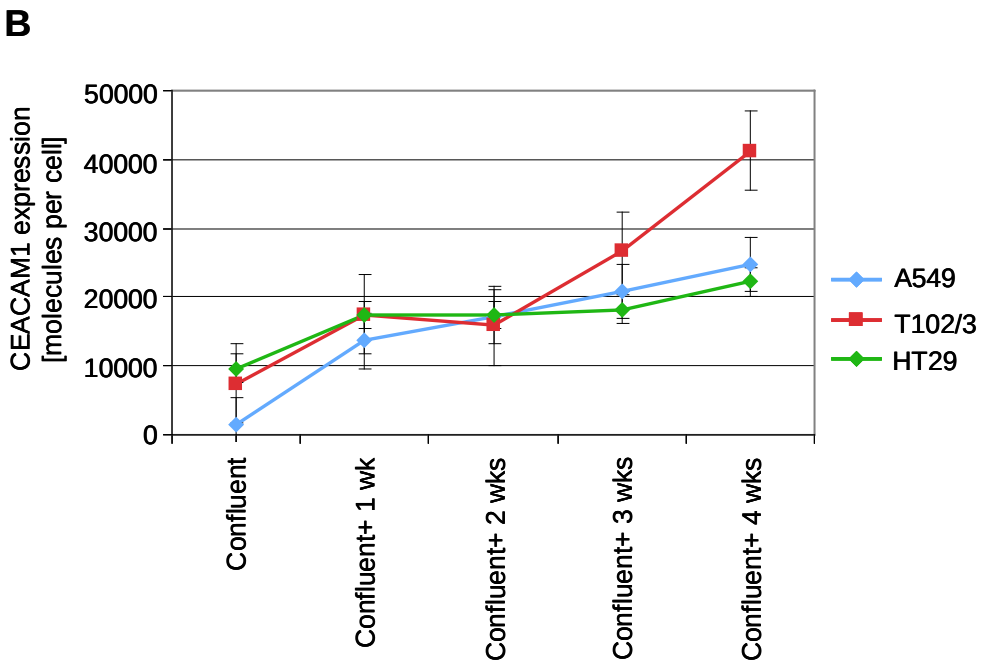
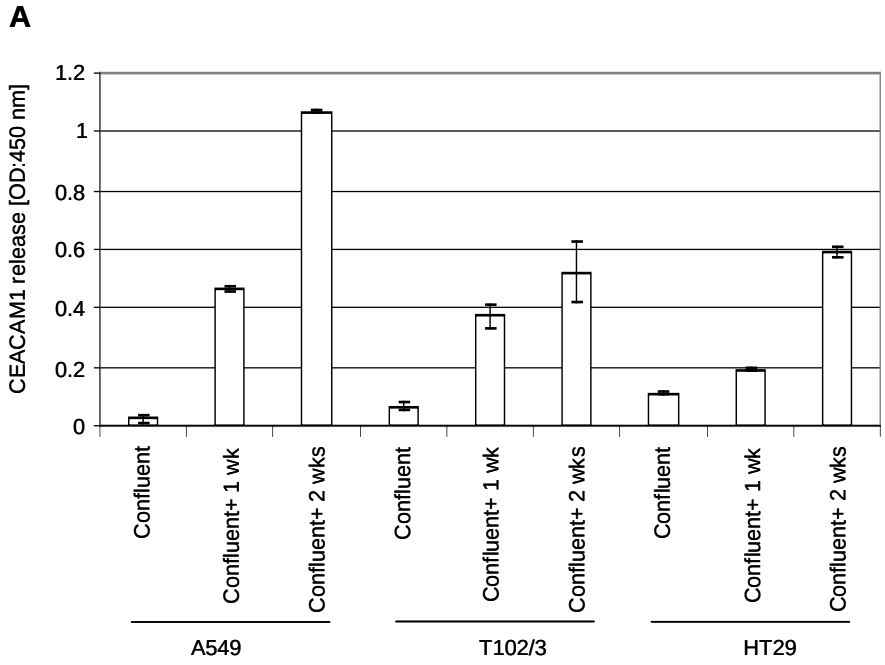


Figure 26: Characterization of the cell surface expression of CEACAM1 in contact inhibited resting cells.
Epithelial cells were collected from different cell growth stages as indicated, were then analyzed by absolute quantitative flow cytometry using the QuiFiKit approach as described in the method part. The values ± SD were determined from triplicates. These are representative data of three separate experiments.

4.3.7 Both full length and truncated CEACAM1 are released into the cell culture supernatant of long term cultured epithelial cells

To determine whether the increased full length and truncated CEACAM1 corresponds with the secreted amount into the cell culture supernatant sandwich ELISA was performed (Figure 27A). The results showed that a very small amount of CEACAM1 was released into the cell culture supernatant of just confluent A549, T102/3 and HT29 cells. However, a significant amount was detectable in confluent plus one week cells. This increased higher in confluent plus two weeks sample. Confluent plus three weeks sample were also analyzed and showed a significant higher amount of CEACAM1 (data not shown). These results showed a

continuous increase of released CEACAM1. To confirm this result immunoprecipitation of the cell culture supernatants was performed (Figure 27B). As shown in figure 15b no band was detected in the cell culture supernatant of just confluent A549 cell whereas to a minor extent, full length as well as truncated CEACAM1 was detected into the cell culture supernatant of confluent T102/3 and HT29 cells. Furthermore, an increasing amount of both full length and truncated CEACAM1 was detected in the cell culture supernatant of confluent plus one week and confluent plus two weeks in all three epithelial cell lines. These results showed that full length as well as truncated CEACAM1 are released and significantly increased when the cells were kept in contact inhibited state.



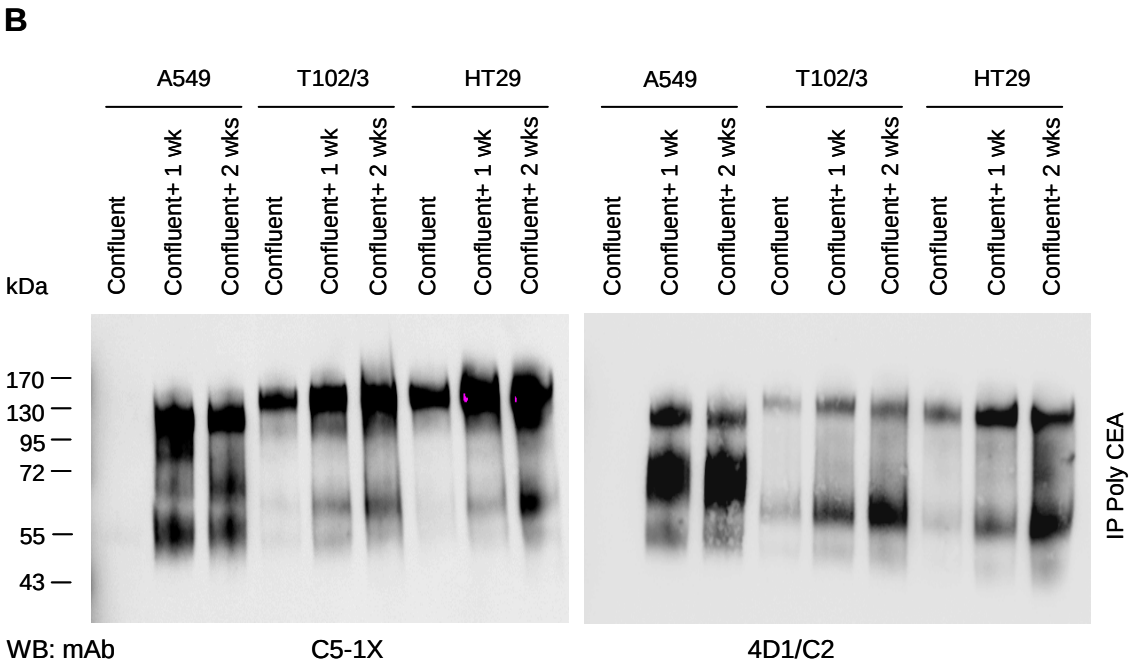


Figure 27: Determination of soluble CEACAM1 released from contact inhibited epithelial cells.
A) For quantification of CEACAM1 released from contact-inhibited epithelial cells, samples were analyzed by sandwich ELISA as described in the methods section. The values ± SD were determined from triplicates. B) Immunoblot analysis for the presence of CEACAM1 released from culture contact-inhibited epithelial cells was done as described in the method section. These are representative data of three separate experiments.

4.3.8 No detection of truncated CEACAM1 on the cell surface

To determine whether truncated CEACAM1 is found on the cell surface, biotinylation assay was performed as described in the method part. An aliquot of the cells were examined for the efficiency of biotinylation by flow cytometry analysis. A control of unbiotinylated cells, and biotinylated cells (0,5 mg/ml and 1 mg/ml) were stained with Cy3-conjugated streptavidin. The measurement showed no staining for the unbiotinylated cells (control) whereas cells biotinylated with 0,5 mg/ml and 1 mg/ml showed a positive reaction median of 3162 and 3751 relative fluorescence, respectively (Figure 28A).

The remaining aliquot of cells were lysed, biotinylated molecules were isolated by affinity purification using streptavidin agarose beads and then subjected to Western blot analysis (Figure 28B). The results using monoclonal antibody 4D1/C2 detected only 120 kDa of full length CEACAM1 but not truncated CEACAM1 in both biotinylated samples. This shows that 72kDa truncated CEACAM1 variant was not biotinylated and thus not present on the cell surface. This experiment was also performed using T102/3 and HT29 and showed similar results (data not shown).

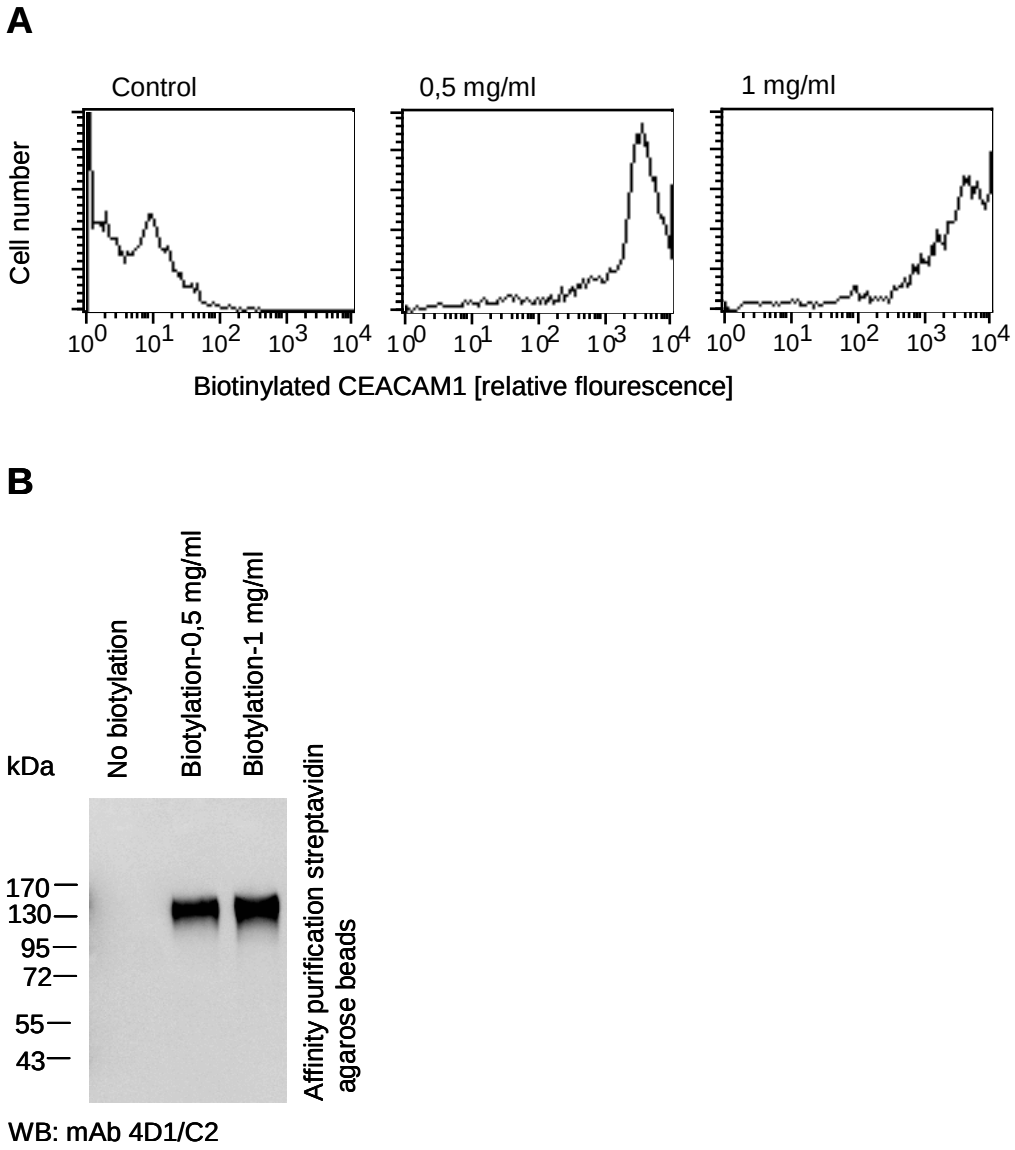


Figure 28: Presence of full length but not truncated CEACAM1 on the cell surface.
A) Cells were biotinylated as described in the method part. Then, they were stained with Cy^{TM}3-conjugated streptavidin and analyzed by flow cytometry analysis. A control of unbiotinylated cells was included. B) To identify cell surface CEACAM1, biotinylated cells were lysed, and subjected to affinity purification using streptavidin agarose beads. Subsequently, the precipitated proteins were analyzed by Western blot using monospecific antibody not against the N-domain of CEACAM1. The data shown are representative of three separate experiments.

4.3.9 Internalization of full length CEACAM1 and appearance of truncated CEACAM1 in turnover processes

Following our results we hypothesized that the full length CEACAM1 would be internalized, and the N-domain and part of the A2 domain together with the cytoplasmic region would be completely digested by e.g. proteases. The remaining part forms the truncated 72 kDa CEACAM1 which accumulates in the cell and finally becomes secreted. To analyze this idea we performed internalization assays on the A549 cells as described in the method part. In principle biotinylation of cell surface receptors allows the examination of receptor internalization, secretion and sometimes recycling process. In this assays, cell surface proteins were first biotinylated on ice. After the removal of the biotin solution and the blockage of unreacted sites, cells were transferred at 37 °C to allow endocytosis of biotinylated CEACAM1. As controls, some cells were not biotinylated while others were biotinylated but not transferred to 37 °C. In line with our hypothesis only CEACAM1 receptors on the surface and those that were internalized as truncated CEACAM1 during 37 °C incubation period remained biotinylated. The biotinylated proteins were isolated with streptavidin agarose beads and CEACAM1 was detected by Western blotting. Our results showed that after 15, 30, 45, and 60 minutes only biotinylated full length CEACAM1 was detectable (Figure 29A). Further increase of internalization time for one hour, three hours, five hours and eight hours respectively produced the similar results that only biotinylated full length CEACAM1 was detectable (Figure 29B). However, when the internalization was increased further the Western blot (Figure 29C) showed a continuous decrease in the amount of biotinylated full length CEACAM1 from day 1, day 2, day 3 and day 4 respectively. In addition, a faint band of truncated CEACAM1 was detected on day 1. One day later (day 2) the amount of truncated CEACAM1 slightly decreased and this continued on day 3 until nothing was detected on day 4. These results demonstrate that the truncated 72 kDa CEACAM1 variant could results from endocytosis of full length CEACAM1. This because previous results demonstrated clearly that truncated CEACAM1 is not localized on the cell surface.

To determine whether the disappearance of both biotinylated full length and truncated CEACAM1 could result in secretion into the cell culture supernatant, affinity purification using strepvidin agarose beads was performed. Interestingly, no biotinylated CEACAM1 was detected in the supernatant (data not shown). This may suggest that once full length CEACAM1 was internalized, truncated CEACAM1 was generated, but almost immediately protease enzymes within the cell could cleave the biotin, resulting in not biotinylated CEACAM1 in the cell culture supernatant.

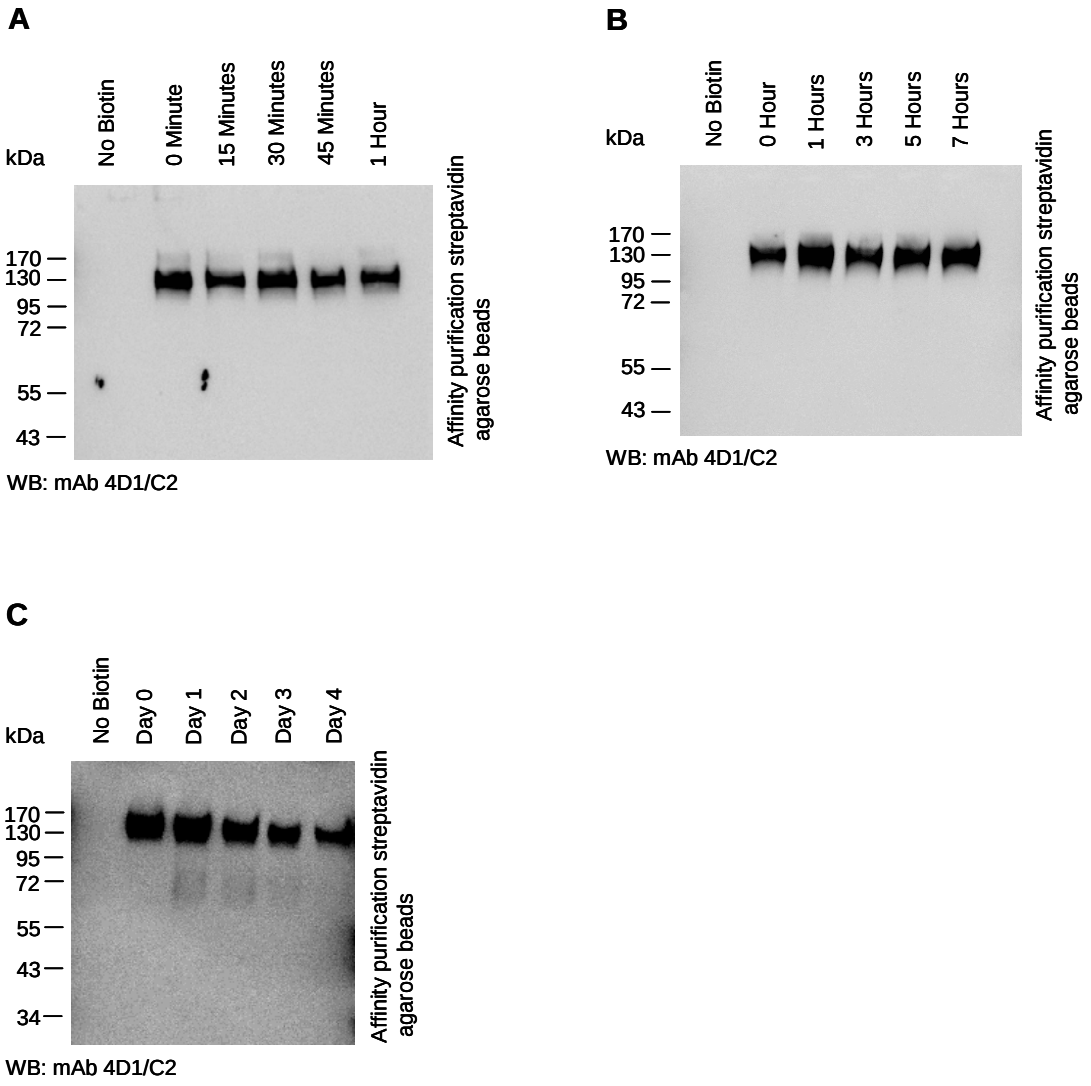


Figure 29: Internalization of full length CEACAM1 and generation of the truncated CEACAM1 variant.
A549 cell were biotinylated with biotin as described in the method section. A) Western blot of biotinylated CEACAM1 after biotin treatment and subsequent culturing for 15 minutes, 30 minutes, 45 minutes, and 1 hour. B) Western blot of biotinylated CEACAM1 after biotin treatment and subsequent culturing for 1 hour, 3 hours, 5 hours and 7 hours. C) Western blot of biotinylated CEACAM1 after biotin treatment and subsequent culturing for 1 day, 2 days, 3 days, and 4 days. Controls of unbiotinylated cells and biotinylated but cultured were included in all experiments. The data shown are representative of three separate experiments.

4.3.10 Regulation of CEACAM1 at the transcription level

Previous results showed an increase of both full length and truncated CEACAM1 in the whole cell lysates of the contacted inhibited epithelial cells. In addition, quantitative analysis of surface CEACAM1 using flow cytometry showed a progressive up-regulation. Total RNA was isolated from the contact inhibited A549 cells to determine whether, CEACAM1 mRNA expression was similar to what was observed with the total protein and the surface expression or whether there were some alterations. Subsequently the mRNA was analyzed by PCR as described in the method section. The primers were designed specific to all CEACAM1 isoforms but with no exon so that only CEACAM1 mRNA would be amplified. GAPDH RNA expression was determined as an internal control to optimize the loading between each time point. The results showed low expression of CEACAM1 mRNA in sparse cells (Figure 30 line 5). Interestingly, a significant expression of CEACAM1 mRNA was observed in just confluent cells (Figure 30 line 1). However, no further change of the CEACAM1 mRNA expression was observed when the cells were kept in contact inhibited state for confluent plus one week, confluent plus two weeks, and confluent plus three weeks, respectively. (Figure 30 line 2, 3, and 4). The expression remained at the same level when cells were grown from confluent to confluent up to three weeks. This experiment was also performed using T102/3 and HT29 and showed similar results (data not shown). These results demonstrate that CEACAM1 is regulated at the translation but not at the transcription level.

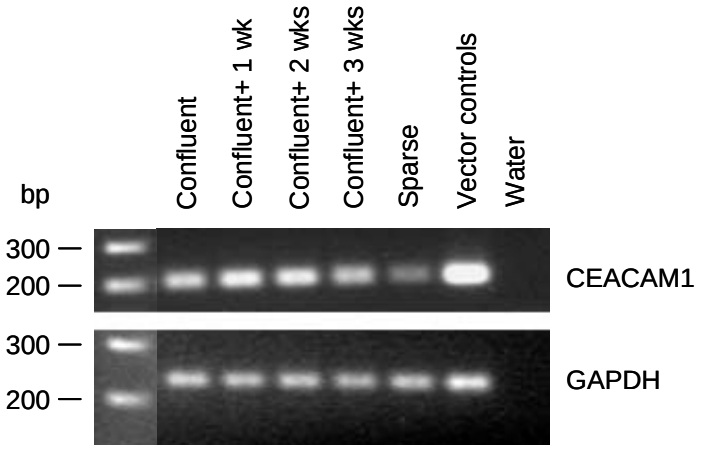


Figure 30: PCR analysis of the CEACAM1 mRNA levels in A549 cells in contact inhibited state.
Vector pcDNA 3.1 containing CEACAM1 and another containing GAPDH were used as positive controls whereas water was used as negative control. The data shown are representative of three separate experiments.

4.4 Characterization of the 95 kDa CEACAM1 variant found in endothelial cells

4.4.1 Analysis of endothelial cells as putative source of truncated CEACAM1

To determine whether endothelial cells could be a further origin of truncated CEACAM1 we analyzed the AS-M.5 an established cortex endothelial cell line. Detection using monoclonal antibodies 18/20 and C5-1X showed 160 kDa bands representing the full length CEACAM1 (Figure 31 left and middle panels). In addition, C5-1X detected a very faint band at 95 kDa. However, detection using monoclonal antibody 4D1/C2 revealed a clear band at 95 kDa and additional faint band of 160 kDa (Figure 31 right panel). A549 cells were included because we found that they express both full length and truncated CEACAM1. These results identified a 95 kDa CEACAM1 variant in endothelial cells which was not detected by an N-domain binding antibody. Thus endothelial cells could be a further source of truncated CEACAM1 found in the urine.

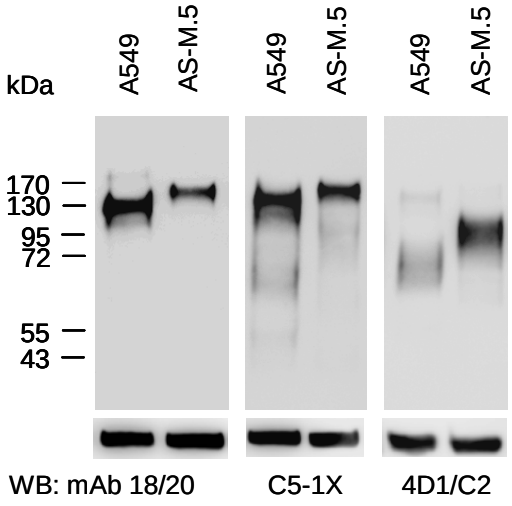


Figure 31: Generation of different variant of truncated CEACAM1 by endothelia versus epithelia. Immunoblot analysis of whole cell lysates from AS-M.5 was performed as described in the method part. Detection utilizing mono specific antibody which binds to N-domain showed full length CEACAM1 whereas using antibodies binding to the A1-B domains or to the linker between B-A2 domains showed both full length and truncated CEACAM1. The detection of beta-actin served as loading control. These are representative data of three separate experiments.

4.4.2 The deglycosylated 95 kDa CEACAM1 variant corresponds to the deglycosylated truncated CEACAM1 variant found in epithelial cells

To investigate whether, the decreased molecular weight of full length CEACAM1 to 95 kDa represent a different glycosylated version of the truncated 72 kDa CEACAM1 variant found in

urine deglycosylation experiments were carried out. The truncated CEACAM1 in epithelial cells is approximately 72 kDa whereas in endothelial it is an N-domain less 95 kDa variant. In parallel, deglycosylation was performed using A549 cells as control. The results showed that both the 95 kDa CEACAM1 found in endothelial as well as the 72 kDa variant found in epithelial reduced to 40 kDa after deglycosylation (Figure 32). Thus, the results demonstrated that the 95 kDa CEACAM1 variant in endothelial cells has apparently a different glycosylation in comparison to that of epithelial cells because when deglycosylated it corresponds to the degylcosylated truncated CEACAM1 variant found in epithelial cells.

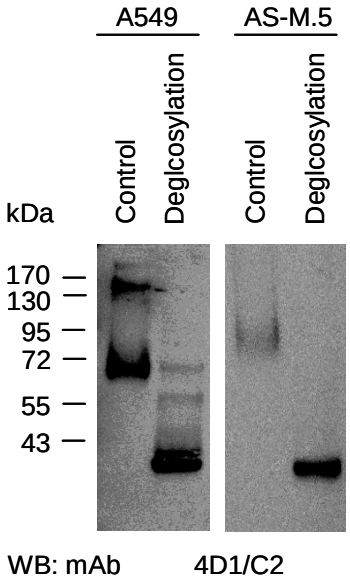


Figure 32: Characterization of non-deglycosylated and deglycosylated CEACAM1 expressed in endothelial and epithelial cells.
Deglycosylation assay was performed using PNGase F enzyme treatment on immunoprecipited CEACAM1. Subsequently, the samples were analyzed by Western blot and detected by a monoclonal anti-CEACAM1. The data shown are representative of three separate experiments.

4.4.3 Truncated CEACAM1 due to protein turn-over processes in endothelial cells

Having learnt from the previous results that truncated CEACAM1 in epithelial cells appeared due to long term culturing of confluent epithelial cells we repeated this approach using the AS-M.5 endothelial cell line. AS-M.5 cells were seeded in 25 mm^2 culture flask, the cell lysates were collected when they reached confluent plus 4 weeks. Results showed very low expression of CEACAM1 at just confluent state (Figure 33). However, the expression was significantly increased in confluent plus four weeks samples. Detection using monoclonal antibody 18/20 showed faint and broad bands at 160 kDa representing the full length CEACAM1 in just confluent and confluent plus four weeks samples respectively (Figure 33 left panel). Furthermore, detection by using C5-1X showed faint and broad bands at 160 kDa representing the full length CEACAM1 in just confluent and confluent plus four weeks

samples respectively (Figure 33 middle panel). In addition C5-1X showed a very weak band at 95 kDa in confluent plus four weeks sample. Interestingly, detection by using the monoclonal antibody 4D1/C2 showed faint and broad bands at 95 kDa in confluent and confluent plus four weeks samples, respectively (Figure 33 right panel). Note, that the 95 kDa was not detected with an N-domain binding antibody confirming that truncated CEACAM1 in endothelial cells lacks N-domain. The results showed that truncated CEACAM1 in endothelial cells appeared due to molecular protein turnover.

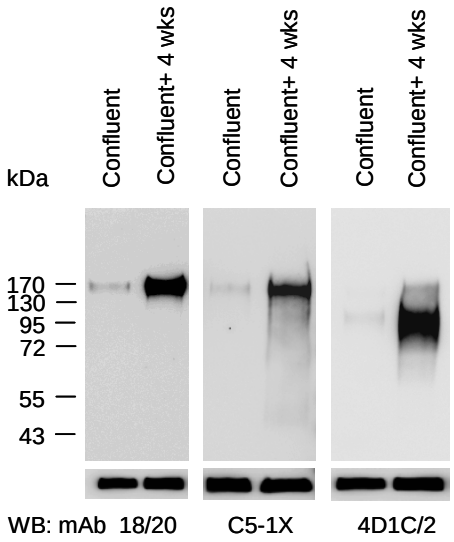


Figure 33: Up regulation of full length and truncated CEACAM1 in contacted-inhibited endothelial cells.
AS-M.5 cells were cultured and there whole cell lysates collected after the cells growth stages as indicated above. Subsequently, samples were analyzed by Western blot and the determination of CEACAM1 was done by using three monoclonal anti-CEACAM1 antibodies. The detection of beta-actin served as loading control. The data shown are representative of three different experiments.

4.4.4 Release of both full length and truncated CEACAM1 are released into the cell culture supernatant of long term cultured endothelial cells

To determine whether, the increase of full length and truncated CEACAM1 in the endothelial cells corresponds with the secreted amount into the culture cell culture supernatant sandwich ELISA was performed (Figure 34A). As shown in figure 34 line 1 no CEACAM1 was detected in the just confluent samples. However, CEACAM1 was a significantly increased to 0.8 ± 0.1 optical density (n=3) in the confluent plus four weeks sample. These results demonstrated that CEACAM1 is released into the cell culture supernatant when the cells were kept in contact inhibited state.

To confirm this results immunoprecipitation of the samples was performed. As shown below no band was detected in the confluent samples. However, detection by using monoclonal antibody 4D1/C2 revealed a weak band at 160 kDa and additional broad band at 95 KDa in

confluent plus four weeks sample (Figure 34 B left panel). In addition, detection using monoclonal antibody C5-1X revealed faint bands at 160 kDa, 95 kDa and at 55 kDa in the same sample (Figure 34 B right panel).

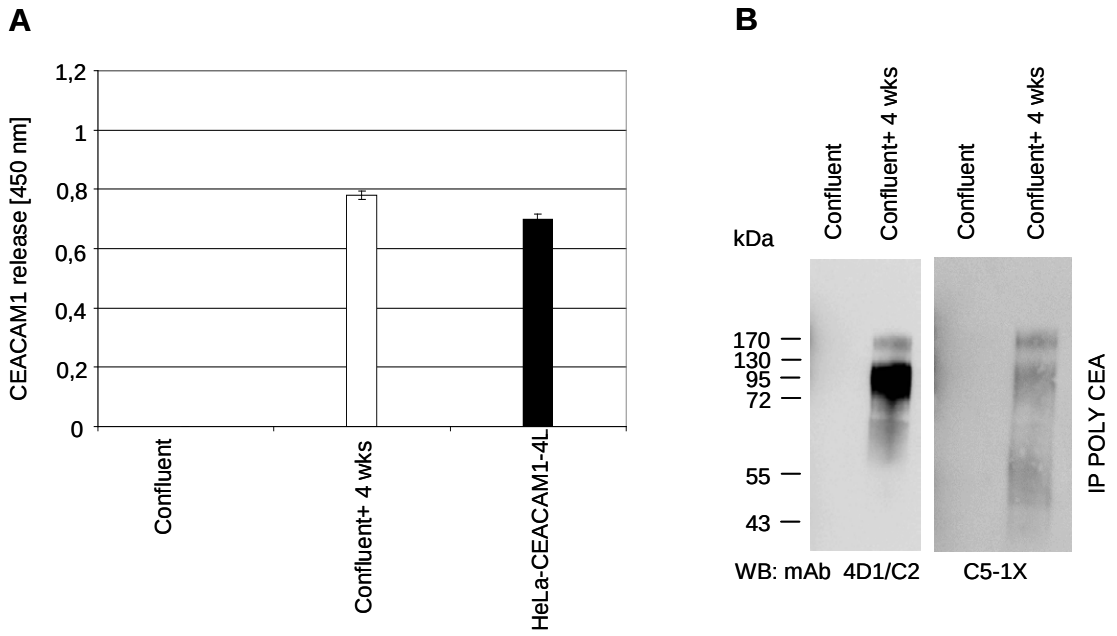


Figure 34: Determination of soluble CEACAM1 released from contact inhibited endothelial cells.
A) For quantification of CEACAM1 released in contact-inhibited endothelial cells, samples were analyzed by sandwich ELISA as described in the method section. As positive control HeLa-CEACAM1-4L cell lysates was included. The values ± SD were determined from triplicates. B) For detection of CEACAM1 released into cell culture supernatant immunobloting analysis was performed as described in the method section. The presented data are representative of three separate experiments.

4.5 Characterization of full length CEACAM1 in urine

4.5.1 Release of full length CEACAM1 from the cells in membrane-bound vesicles

We found that only truncated CEACAM1 is present in urine whereas epithelial and endothelial cells released both full length as well as truncated CEACAM1. To elucidate this discrepancy we analyzed the pellets obtained from urine after centrifugation. The urine samples from patients suffering from bladder cancer were subjected to centrifugation as described in the method part. The pellets obtained were then analysed by Western blot analysis. The results showed broad bands at 120 kDa indicating high amount of full legth CEACAM1 in the pellets (Figure 35). However, no truncated CEACAM1 was detected. The results shows that the full length CEACAM1 is usually released as membrane-bound whereas truncated CEACAM1 are soluble.

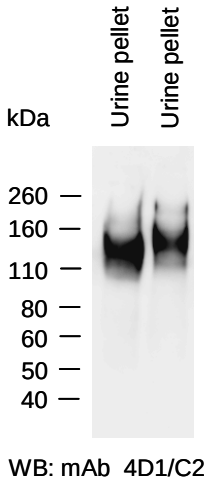


Figure 35: Detection of full length as well as truncated CEACAM1 found in urine pellets.
The urine samples were subjected to centrifugation as described in the method section. Subsequently, the pellets ware collected and analyzed by Western blot as described in the method section. The blot was developed using monospecific Anti-CEACAM1 antibody.

5. Discussion

Although it was published that no CEACAM1 is released into human urine (Draberova et al., 2000; Svenberg, 1976b), soluble forms have recently been reported to be present in urine of healthy donors. Interestingly, its urine concentration was significantly higher in patients suffering from bladder cancer (Tilki et al., 2009). However, the soluble CEACAM1 found in urine was not yet sufficiently characterized. Therefore, this study focused on the protein biochemical characterization of CEACAM1 found in urine, particularly on mechanisms leading to generation of this CEACAM1 form and the determination of its putative cellular origin. Furthermore, we wanted to learn more about the cause leading to the CEACAM1 secretion and the detailed mechanism behind these processes.

5.1 Soluble CEACAM1 is present in human urine

Normally CEACAM1 is expressed on the cell surface of various cell types. It has been shown that it is expressed on epithelial cells, and angiogenicly activated endothelial cells (Horst and Wagener, 2004; Prall et al., 1996; Ergun et al., 2000b; Kilic et al., 2005). It is also expressed on various hematopoietic cell types such as granulocytes, B cells, T cells, natural killer (NK) cells and dendritic cells (Kammerer et al., 1998; Khan et al., 1993; Moller et al., 1996b; Singer et al., 2002; Watt et al., 1991). Beside the transmembrane-bound form soluble CEACAM1 variants has been reported. Presence of soluble CEACAM1 has been reported in the serum of healthy donors (Svenberg et al., 1981). Furthermore, increased serum levels of soluble CEACAM1 were observed in patients with liver diseases such as obstructive jaundice, hepatitis A or B, and cytomegalovirus-induced hepatitis (Draberova et al., 2000; Svenberg et al., 1979). In addition, soluble CEACAM1 has been found in human bile (Svenberg, 1976b). In contrast to these literature reporting the existence of soluble CEACAM1 it was reported that soluble CEACAM1 was not detectable in urine (Draberova et al., 2000). On contrary, our group recently found CEACAM1 in urine of healthy donors and at significantly higher concentration in urine samples of patients with bladder carcinoma (Tilki et al., 2009). In addition, Tilki et al., 2009 observed CEACAM1 bands with 120 kDa and another with approximately 72 kDa. However, the origin of soluble CEACAM1 was not investigated. To further investigate this CEACAM1 form we first determined the concentration of CEACAM1 in urine of healthy donors in comparison with the well characterized concentration of CEA. CEA in urine was previously reported to be in the range of 7-11 ng/ml (Guinan et al., 1974). Our results showed concentration of 7 ng/ml ± 5 ng/ml (n=12) for CEA (Figure 11 left panel). Surprisingly, the concentration of CEACAM1 was significantly higher having 200 ng/ml ± 110 ng/ml (n=16) (Figure 11 right panel). However, CEACAM1 showed a very large standard deviation with the highest been ten fold higher than the smallest. This may be due to individual differences arising from diet, and also it may be due to differences in the time

urine was collected (24-hours or morning collection). To confirm the presence of CEACAM1 in urine immunobloting analysis was performed. Our results revealed a significant band with molecular weight of 72 kDa band (Figure 12 line 4) which confirms the findings published in a recent study (Tilki et al., 2009). On the contrary, we did not detect the 120 kDa CEACAM1 form as shown by Tilki et al.

Next, we analyzed if CEACAM1 found in urine is soluble or membrane-bound. This was performed using ultracentrifugation and Triton X-114 phase separation assays. Our results showed that main part of CEACAM1 was detected in the hydrophilic fraction and minor part in the hydrophobic fraction (Figure 13A and B). Therefore, approximately 90% of the CEACAM1 found in urine represent soluble and not membrane-anchored.

5.1.1 72 kDa CEACAM1 variant found in urine does not represent a deglycosyleted variant of full length CEACAM1

Depending on the cell type the molecular weight of CEACAM1 can be 120-160 kDa due to differences in glycosylation (Kannicht et al., 1999; Lucka et al., 2005). For example, CEACAM1 on PMNs cells have a molecular weight of 160 kDa (Lucka et al., 2005) whereas epithelial and endothelial cells have 120 kDa and 140 kDa respectively (Bamberger et al., 1998; Prall et al., 1996). However, due to the protein marker we used in this study sometimes the molecular weight was higher. Therefore, in this study we decided to use 120 kDa to represent full length CEACAM1.

To determine whether the reduction from 120 kDa to 72 kDa may occur due to glycosylation differences, CEACAM1 in urine was subjected to deglycosylation using PNGase enzymes. Deglycosylation of the 72 KDa CEACAM1 precipitated from urine resulted in a 40 kDa band (Figure 14A right panel). Furthermore, deglycosylation of the human CEACAM1-Fc reduced its molecular weight from 120 kDa to 75 kDa (Figure 14A left panel). This clearly demonstrated that the 72 kDa CEACAM1 detected in urine is glycosylated and does not represents a deglycosylated variant of full length CEACAM1. Calculating from our results the reduction of full length CEACAM1 from 120 kDa to 75 kDa showed that 62.5% consists of sugar. Whereas truncated CEACAM1 consist of 55.5% sugar. These corresponded with what is known from the literature that more than half of the molecular weight of CEACAM1 consists of carbohydrates (Lucka et al., 2005).

Beside proteins been secreted from the body via urine they can also be secreted through feces. This led us to analyse CEACAM1 in comparison with CEA in feces. Immunobloting analyses on feces samples revealed a strong band with molecular weight of 40 kDa (Figure 14B right panel). It is likely that CEACAM1 detected in feces is deglycosylated for it corresponds with the deglycosylated CEACAM1 in urine. However, CEACAM5 in feces revealed most prominent band at 180 kDa (Figure 14B left panel). This prominent band corresponded with previous report (Kuroki et al., 1981). It is likely that the deglycosylation of

CEACAM1 in feces is carried out by digestive enzymes or by microorganisms in the colon. Taken together our results clearly demonstrated that the reduction of molecular weight from 120 kDa to 72 kDa is not due to differences in glycosylation of CEACAM1.

5.1.2 Soluble CEACAM1 found in urine lacks the N-terminal domain and part of A2 domain

Next, we analyzed if the reduced molecular weight appears due to different number of domains as observed in CEACAM1 isoforms. For example, CEACAM1-4 isoforms have the A2 domain which is missing in the CEACAM1-3 isoforms. We established a different sandwich ELISA to distinguish between the N-terminal domain, A1B1, A2, and cytoplasmic domain respectively. Our results revealed that the 72 kDa CEACAM1 variant found in urine does not contain the N-domain, part of the A2 domain and cytoplasmic tail (Figure 15A). Therefore, CEACAM1 in urine represent a truncated variant consisting of the A1, B domains and a part of the A2 domain. Because all eleven expressed isoforms of CEACAM1 contain the N-domain, the truncated CEACAM1 variant can not be one of the splice variants. Therefore, we present here for the first time a novel truncated variant of CEACAM1.

These clearly demonstrate that Draberova et al., were not able to detect CEACAM1 in urine because they utilized an antibody that binds to the N-domain which according to our results it is missing. On other hand we detected it because we used antibody that binds at the linker between B and A2 domains.

We further performed epitope mapping of CEACAM1 found in hydrophilic fraction of urine after ultracentrifugation. Our results confirmed that the CEACAM1 in the hydrophilic fraction does not contain the N-domain, part of the A2 domain and cytoplasmic tail (Figure 15B). Taken together our results clearly demonstrate that CEACAM1 found in urine is a truncated variant and this variant might originate from hematopoetic, epithelial and endothelial cells.

5.2 Granulocytes are not likely the source of truncated CEACAM1

Human polymorphonuclear leukocytes (PMNs; neutrophils or granulocytes) are critical for innate host defense and comprise the single greatest cellular component of the immune system. Approximately 60% of all human leukocytes are granulocytes (Bainton et al., 1971). PMNs cells have the broadest CEACAM1 expression. This led us to investigate whether PMNs cells would be the likely source of truncated CEACAM1. Western blot analysis by applying two monoclonal antibodies 18/20 (binds the N-domain) and 4D1/C2 (binds the linker between B and A2 domains) revealed that PMNs cells express only the full length but not the truncated CEACAM1 (Figure 16).

Having shown that PMN cells do not endogenously express truncated CEACAM1 we thought that it may be generated when they under spontaneous apoptosis. It is important that neutrophils undergo constitutive (spontaneous) apoptosis as a mechanism to facilitate normal cell turnover and immune system homeostasis (Haslett et al., 1991). Normally, granulocytes are short-lived cells with a half-life of only 6-20 hours in the circulation, after which they undergo apoptosis. Previous studies reported that healthy donors produce and destroy 180 x 107 granulocytes per day (Haslett et al., 1991; ATHENS et al., 1961). Immunoblot analysis of the cell lysates after spontaneous apoptosis revealed full length CEACAM1 but not truncated CEACAM1 (Figure 17A lines 1 and 2). Furthermore, analysis of the supernatant obtained indicated that spontaneous apoptosis did not lead to secretion of truncated CEACAM1 but rather a small fraction of full length CEACAM1 (Figure 17A line 3).

These results were expanded using a Fas ligand (FasL) which is a potent inducer of apoptosis in granulocytes (Simon, 2003). The interaction of FasL with its receptor results in the activation of a defined downstream caspase cascade, which amplifies the apoptotic process (Sharma et al., 2000). Our results revealed that truncated CEACAM1 was not detected in the cell lysates even after induced apoptosis (Figure 17B line 1 and 2). Furthermore, truncated CEACAM1 was not detected in the supernatant (Figure 17B line 3). Therefore, we conclude that truncated CEACAM1 is not expressed or secreted from granulocytes due to apoptosis processes. However, our findings seem to be in contrast to previous report that apoptosis on epithelial cells leads to cleavage of CEACAM1 (Nittka et al., 2008). These different results may be based on the fact that we used granulocytes where as Nittka et al. utilized epithelial cell lines. Therefore, suggesting that the role of CEACAM1 during apoptosis processes depended on the cells type.

It is commonly accepted that PMNs cells which are recruited to the site of infection or senescent PMN cells, eventually succumb to apoptosis and are swiftly ingested by macrophages (Savill et al., 1989). The clearance of PMNs cells by macrophages is a crucial process in events such as homeostasis, wound healing and tissue regeneration (Hart et al.,

2008). Since PMN are short-lived cells their recognition and clearance must be extremely efficient. Apoptotic PMN cells that would not be removed by macrophages undergo necrosis leading to the release of their cytotoxic granular contents, and thus damaging the surrounding tissues (Haslett et al., 1994). Clearance of this apoptotic cells requires highly specific signals exposed on the surface (Lauber et al., 2004). In comparison to healthy cells, apoptotic cells contain altered proteins, lipids, and carbohydrates on their surface (Hart et al., 2008). The best characterized of such molecule is the phospolipid phoshatidlylserine (PS) which relocates from the inner to the outer surface of the plasma membrane (Fadok et al., 1992; Martin et al., 1995). This facilitates the recognition and ingestion of apoptotic PMNs cells.

To mimic these physiological processes, PMNs cells and macrophages were co-cultured to determine whether truncated CEACAM1 do not appear due to these processes. Immunobloting analysis of the cell lysates after co-culture revealed that truncated CEACAM1 appears not due to ingestion of PMN by macrophages (Figure 18 lines 1, 2, 4, 5, 7, and 8). In addition, no truncated CEACAM1 was detected in the cell culture supernatant (Figure 18 lines 3, 6 and 9). These results clearly demonstrate that truncated CEACAM1 found in urine is probably not produced or secreted from ingestion of apoptotic PMNs cells by macrophages.

5.3 Epithelial cells are the likely source of truncated CEACAM1

Epithelial cells are another type of cells that express significant amount of CEACAM1. This led us to investigate if epithelial cells are likely the source of truncated CEACAM1. Immunoprecipitation of CEACAM1 was performed on different epithelial cells (A549, T102/3, HT29, and HeLa-CEACAM1) using a monospecific antibody 4D1/C2. Subsequently, Western blot analysis using monoclonal antibody 18/20 revealed bands with molecular weight of 120 kDa which corresponds to full length CEACAM1 but no truncated CEACAM1 (Figure 19A). However, detection by using the monoclonal antibody 4D1/C2 revealed bands with molecular weight of 120 kDa and 72 kDa (Figure 19 B). These results demonstrated for the first time that epithelial cells are likely the source of truncated CEACAM1. Furthermore, our results clearly demonstrated that truncated CEACAM1 was found in both endogenously and transfected CEACAM1 expressing epithelial cells. Therefore, we concluded that epithelial cells are likely source of truncated CEACAM1 variant. However, it was important to analyze if this reduction of molecular weight from 120 kDa to 72 kDa was due to different glycosylation.

5.3.1 72 kDa CEACAM1 variant found in epithelial cells does not represent a deglycosylated variant of full length CEACAM1

Deglycosylation assay was performed to determine whether the reduction of molecular weight from 120 kDa to 72 kDa was due to differences in glycosylation of CEACAM1. First of all CEACAM1 in epithelial cells was precipitated using monoclonal antibody B3 (binds A1B-domains) then subjected to deglycosylation. Subsequently, Western blot analysis using monoclonal antibody 4D1/C2 revealed that deglycosylation of the full length CEACAM1 reduced its molecular weight from 120 kDa to 75 kDa whereas the 72 kDa truncated CEACAM1 variant reduced to 40 kDa (Figure 20). This result clearly demonstrates that the 72 kDa CEACAM1 variant does not represent a deglycosylated variant of full length CEACAM1. In addition, our results on the reduction of full length CEACAM1 from 120 KDa to approximately 75 kDa after deglycosylation are in accordance with previous reports (Nittka et al., 2008; Comegys et al., 2004). Furthermore, deglycosylated CEACAM1 bands were detected as weak bands suggesting the importance of sugar in antibody binding and once the sugars are removed the antigen-antibody interaction was weakend.

5.3.2 CEACAM1 found in epithelial cells also lacks N-domain

Next we analyzed whether the 72 kDa CEACAM1 variant expressed in epithelial cells lacks the N-domain as in case of CEACAM1 found in urine. This was addressed by epitope mapping using immunoblot analysis. Immunoprecipitation was performed using monoclonal antibodies B3 and 4D1/C2. Subsequently, Western blot using monoclonal antibody 18/20 revealed bands with molecular weight of 120 kDa (Figure 21 left panel). Thus, indicating that

the 72 kDa CEACAM1 variant does not have N-domain. However, detection by using the monoclonal antibody 4D1/C2 revealed two bans with molecular weight of 120 kDa and 72 kDa (Figure 21 right panel). Taken together our results clearly demonstrated that the 72 kDa CEACAM1 found in epithelial cells lacks the N-domain. Therefore, similar to epitope mapping of CEACAM1 found in urine it clearly shows that the 72 kDa CEACAM1 is a truncated variant of CEACAM1 which do not have the N-domain.

5.3.3 Truncated CEACAM1 appears not due apoptosis process

It was recently reported that induction of apoptosis on epithelial cells leads to cleavage of CEACAM1 (Nittka et al., 2008). This led us to determine if truncated CEACAM1 appears due to apoptosis. We therefore performed a detailed analysis of apoptosis using apoptotic inducing agents such as actinomycin, campthecin, cycloheximide, dexamethasone and etoposide. Because all apoptotic inducing agents had almost similar results in this study we only use the results of actinomycin. Flow cytometric analysis of Annexin V-FITC/Propidium Iodide (PI) stainings showed that actinomycin led to a significant cell death showing approximately 5% cell survival after 24 hours in A549 cells (Figure 22A and B). Western blot analysis revealed full length but not truncated CEACAM1 variant (Figure 22 C). In addition, in our investigation we did not detect a 14 kDa on fragment of N-domain as previously report by Nittka et al. These results clearly demonstrated that in our experiments truncated CEACAM1 does not appear due to apoptosis.

5.3.4 Truncated CEACAM1 variant appears not due to down regulation of full length CEACAM1 when contact-inhibited resting epithelial cells enter the proliferation

It was recently, reported that A549 (human alveolar type 11) epithelial cells that had reached confluency did not express CEACAM1 but increased as cell entered the post-confluent growth arrest state reaching to high expression at day seven (Singer et al., 2010). In addition Singer et al., showed a significant down regulation of cell surface CEACAM1 when contact inhibited cells are entering the proliferation phase. Thus, it was tempting to speculate that this down regulation leads to the truncated CEACAM1 variant. Surprisingly, our results revealed a significant down regulation of truncated CEACAM1 with complete disappearance after three days when contact inhibited cells began to proliferate (Figure 23A). We show that although the full length CEACAM1 expression disappeared no increased amount of truncated CEACAM1 was detectable. In addition our results revealed for the first time a down regulation of truncated CEACAM1. Further we investigated whether this downregulation of full length and truncated CEACAM1 leads to an increase in the supernatant. Sandwich ELISA analysis of the supernatants showed very small amount of CEACAM1 released in post-confluent cells (day 0) whereas nothing was detected in proliferating cells (Figure 23B). In addition, Western blot analysis revealed no full length as well as truncated CEACAM1 in

the cell culture supernatant (data not shown). Therefore, we concluded that truncated CEACAM1 does not appear or secreted from epithelial cells due to down regulation of full length CEACAM1 expression when contact-inhibited cells entered the proliferative stage. The down regulation of full length and truncated CEACAM1 implies that proliferating cells needs a lot of energy. Thus, they do not release full length or truncated CEACAM1 but rather every thing is reused. Therefore, this is a type of CEACAM1 turnover process whereby the cell reuses every from CEACAM1 which includes amino acids and sugars.

We next quantified CEACAM1 expression in different cell growth phases of epithelial cells, using quantitative flow cytometry approach. The cells were stained with monoclonal antibody 18/20 and the level of CEACAM1 expression was determined in reference to calibration beads conjugated with a known amount of antigen for secondary antigen. In log phase of HT29 and T102/3 cells a significant expression of approximately 8,000 ± 1500 (n=4) molecules per cell was observed where as in case of A549 cells no CEACAM1 expression on the surface of was detected. However, a signification up-regulation with approximately 18,000 ± 4000 (n=4) molecules per cell was observed when all cell types were kept at confluency (Figure 24).

5.3.5 Truncated CEACAM1 appears in significantly amount in contact inhibited resting epithelial cells

Contact-inhibition is the process of arresting cell growth when cells come close to each other. As a result normal cells stop proliferation when they form a monolayer in a culture dish. Cancer cells mostly do not arrest their growth when they fill a culture dish but continue to proliferate, piling up on top of each other and forming multilayered foci. The loss of contact-inhibition is a hallmark of malignant cancer cells, leading to higher proliferation rate (Hanahan and Weinberg, 2000). Recently, it was reported that A549 cells that had reached confluence did not express CEACAM1 but the expression increased as cell entered the post-confluent contact inhibited growth stage (Singer et al., 2010). This led us to investigate whether the increase of full length CEACAM1 during post-confluent phase would lead to appearance of truncated CEACAM1. We therefore analyzed different epithelial cell lysates (A549, T102/3, and HT29) when cells were confluent, confluent plus one week, confluent plus two weeks, and confluent plus three weeks. Western blot analysis of A549 cells (Figure 25A right panel) using monoclonal antibody 18/20 showed no truncated CEACAM1 any of the samples. Thus, confirming that truncated CEACAM1 does not have N-domain. However, using monoclonal antibodies C5-1X and 4D1/C2 weak bands of truncated CEACAM1 were observed in the confluent samples (Figure 25A lines 2 and 3). Interestingly, it was observed that the truncated CEACAM1 continuously increased as the cell were kept in contact inhibition growth phase (Figure 25A middle and left panels). Western blot analysis of T102/3 (Figure 25B) and HT29 (Figure 25C) revealed the same increase of full length and truncated

CEACAM1 in contact inhibited cells. Furthermore, detection by using the three monoclonal antibodies revealed a continuous increase of full length CEACAM1 in all three epithelial cells (Figure 25A, B and C). This finding was expanded by quantitative flow cytometric analysis of cell surface CEACAM1. The CEACAM1 expression was analyzed as cells were just confluent, confluent plus one week, confluent plus two weeks, and confluent plus three weeks (Figure 26). The results showed low expression of CEACAM1 in just confluent cells. However, the expression progressively increased attaining approximately 25,000 ± 2,200 (n=3), 41,000 ± 5,030 (n=3), and 22,000 ± 1,850 (n=3) molecules per cell in A549, T102/3 and HT29 cells, respectively.

Taken together, our results revealed for the first time that truncated appeared in contact-inhibited cells and significantly increased as were cell kept longer in contact inhibited growth state.

5.3.6 Contact inhibited resting epithelial cells, but not proliferating cells release truncated CEACAM1

Next, we analyzed using sandwich ELISA the cell culture supernatants obtained from epithelial cells kept in contact inhibited state. Our results revealed a continuous increases of released CEACAM1 (Figure 27A). Using immunobloting analysis no truncated CEACAM1 was observed in confluent samples (Figure 27B lines 1, 4, 7, 10, 13, and 16). This clearly demonstrates that truncated CEACAM1 is not released during cell proliferation. However, significant amount of truncated CEACAM1 was observed in samples of cells with one week and two weeks after conflueny (Figure 27B). These clearly demonstrate that truncated CEACAM1 variant is released from contact inhibited resting epithelial cells. In addition it was significantly increased as contact inhibited cells well kept longer. Furthermore, our results revealed an increasing amount full length CEACAM1 (Figure 27B).Taken together our results demonstrated that full length as well as truncated CEACAM1 is significantly released from contacted inhibited cells. In comparison to our earlier results obtained from urine analyses these findings show not only the truncated CEACAM1 is released. To elucidate this discrepancy, additional studies were done using urine as explained in section (4.5). However, the functions of transmembrane-bound and soluble full length CEACAM1 are well known. The cytoplasmic domain of CEACAM1 contains two phosphorylable tyrosine residues in the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that are important in the regulation of signal transduction (Obrink, 1997a). After tyrosine phosphorylation, CEACAM1–4L can bind and activate src-family tyrosine kinases (Brummer et al., 1995; Skubitz et al., 1995) as well as the SH2-domain-containing protein tyrosine phosphatases SHP1 and SHP2 (Beauchemin et al., 1997). In addition, an increased intracellular calcium concentration leads to binding of calmodulin to the cytoplasmic domains of both the L and the S isoforms, which influences CEACAM1 dimerization (Edlund et al., 1996a). Interactions of CEACAM1 with the

actin filament system also have been observed (Da Silva-Azevedo and Reutter, 1999; Hunter et al., 1994). The cytoplasmic domain of CEACAM1-L confers on FcyR11B the capacity to inhibit B-cell antigen receptors (BCR) (Chen et al., 2001). Furthermore, it has been reported that tumor suppressive effect of CEACAM1 depends on the presence of its cytoplasmic domain (Izzi et al., 1999; Turbide et al., 1997). Cytoplasmic domain of CEACAM1 is also essential during formation of insulin-insulin receptor (IR) complex (Choice et al., 1998; Najjar et al., 1998). Therefore lack of cytoplasmic domain by the truncated CEACAM1 means it can not to be involved in the above mentioned signal transductions involving cytoplamic domain.

It is well established that CEACAM1 mediates intercellular interactions using its N-domain (Watt et al., 2001; Markel et al., 2004a; Stern et al., 2005; Klaile et al., 2009a). In addition, it was shown that the *trans*-homophilic CEACAM1 binding induces *cis*-dimerization by an allosteric mechanism transmitted via the N-domain (Muller et al., 2009a). Furthermore, trans-binding between the N-domains changes CEACAM1 interactions with microcluster (Klaile et al., 2009a). Studies have shown that Opa proteins expressed by *Neisseria gonorrhoeae* and *Neisseria meningitides*, bind specifically to the N-domain of CEACAM1 (Gray-Owen et al., 1997; Bos et al., 1999). The binding UspA1 proteins expressed by *Moraxella catarrhalis* on CEACAM1 also occurs through the N-domain (Hill and Virji, 2003). Other studies have shown that of *Escherichia coli* and *salmonella* strains expressing Dr family of adhesins bind CEACAM1 on the N-domain (Korotkova et al., 2006). In addition, ligation of CEACAM1 with monoclonal antibody addressing N-domain stimulates β2-integrin-dependent neutral aggregation (Kuijpers et al., 1993) and adherence of neutrophils to HUVECs (human umbilical vein endothelial cells) (Kuijpers et al., 1992). Furthermore, synthetic peptides containing sequences of the N-domain of CEACAM1 can initiate signal transduction in neutrophils (Skubitz et al., 2001; Skubitz and Skubitz, 2010). It has been shown that N-domain is crucial for enhancing insulin endocytosis (Soni et al., 1999). Our results have clearly demonstrated that, truncated CEACAM1 lacks the N-domain which is essential in many CEACAM1 functions. Therefore, lack of N-domain by the truncated CEACAM1 means it can not be involved in the any CEACAM1 function involving N-domain.

Several functions of soluble CEACAM1 have been reported. Recently, soluble full length CEACAM1 was shown to exhibit angiogenic properties *in vitro* and *in vivo* (Ergun et al., 2000b). It was demonstrated that CEACAM1 mediated inhibition of NK cell killing activity is specifically abrogated in the presence of soluble full length CEACAM1 (Markel et al., 2004b). In addition CEACAM1 can also act a co-inhibitor of T cells receptor (TCR) responses when bound with soluble full length CEACAM1-Fc (Chen et al., 2004). However, soluble truncated CEACAM1 has never been reported. We suggest that lack of N-terminal and cytoplamic domains by the soluble truncated CEACAM1 imply it can not involved in any CEACAM1

function. Furthermore, it can not be used a ligand due to lack of N-domain for example CEACAM1-ΔN-Fc (without N-domain) constructs are mostly used as a negative control. Therefore, we suggest that this is a type of CEACAM1 turnover process in epithelial cells when they do not need all CEACAM1 thus removes the cytoplasmic and the N-domains making it not dangerous.

5.3.7 Truncated CEACAM1 is generated by internalization mechanism of full length CEACAM1 as molecular turnover process

Some studies have shown that transmembrane proteins can not sufficiently be degraded by proteinases as long as they are membrane bound. For example the urokinase receptor uPAR (Montuori et al., 2005) and CD44 (Lammich et al., 2002) are cleaved within the cell membrane. Then, these fragments are internalized and moved to the lysosomes for degradation. In confluent contact-inhibited cells the truncated CEACAM1 accumulated in the cell as well as in the supernatant. Biotinylation studies should help to reveal if truncated CEACAM1 comes from the surface. A549 cells were incubated with sulfo-NHS-LS biotin one ice for one hour. Then immediately the biotinylated proteins were isolated by affinity purification using streptavidin agarose beads. Subsequently Western blot analysis revealed that only full length CEACAM1 was biotinylated but not truncated CEACAM1 (Figure 28B). Therefore, confirming that truncated CEACAM1 is not localized on the surface due to lack of the cytoplasmic domain. Next we analyzed the generation of truncated CEACAM1 by culturing biotinylated A549 cells at 37°C to allow the biotinylated molecules to be internalized. Western blot analysis of cell lysates after culturing for 15, 30, 45, and 60 minutes revealed only full length CEACAM but not truncated CEACAM1 (Figure 29A). Furthermore, increase of culturing time to one hour, three hours, five hours and 8 hours respectively demonstrated similar results (Figure 29B). The increase of culturing time to 24 hours demonstrated significant band at 120 kDa and a week band at 72 kDa. However, as the cells were cultured longer these bands became less and in day 4 no truncated CEACAM1 was visible (Figure 29C). To determine whether the disappearance of both biotinylated full length and truncated CEACAM1 could result to secretion into the cell culture supernatant, affinity purification using streptavidin agarose beads was performed. Interestingly, no biotinylated CEACAM1 was observed in the supernatant (data not shown). This may suggest that once full length CEACAM1 was internalized, truncated CEACAM1 was generated, and immediately protease enzymes within the cells cleave the biotin out, resulting into not biotinylated CEACAM1 in the cell culture supernatant. Additional studies are needed to investigate the pathway involved in the internalization processes.

5.3.8 CEACAM1 is regulated at translational but not transcriptional level in epithelial cell during contact-inhibited growth phase

Based on the a fore mentioned findings we next we investigated if CEACAM1 mRNA varied during contact-inhibited grow phase and if it is similar to the continuous increase as observed on full length and truncated CEACAM1. Our results demonstrated that the level of CEACAM1 mRNA remained constant in contact-inhibited epithelial cells (Figure 30). Furthermore no changes on the mRNA level were observed even after three weeks when cells were kept in the contact inhibited state. This suggests that the significant increase of full length and truncated CEACAM1 in contact-inhibited cells may be due to increased translation of protein but not due to transcription as mRNA levels remained steady state. However, truncated CEACAM1 may come from new synthesis. This happens when the cells constitutively produce CEACAM1 but the cell does not need all CEACAM1 produced, thus it degraded it to truncated CEACAM1 before reaching the cell surface. These may be the reason why we were not able to observe truncated variants of CEACAM1 after biotinylation.

5.4 Endothelial cells represent an additional source of truncated CEACAM1

Beside leukocytes and epithelial cells CEACAM1 is also expressed on angiogenicly activated vascular and lymphatic endothelial cells (Ergun et al., 2000b; Kilic et al., 2005; Kilic et al., 2007). Endothelial cells represent a special form of epithelial cells thus it was tempting to speculate that also these cells might express truncated CEACAM1. To this aim a cortex endothelial cell line (AS-M.5) was used in our analysis. Our results revealed that full length CEACAM1 from epithelial cells was lower than that detected in endothelial cells (Figure 31). This results are in accordance with several reports indicating that molecular weight varies due to the level of CEACAM1 glycosylation depending on the cell type (Kannicht et al., 1999; Lucka et al., 2005). Interestingly it was noted that immunobloting analyses using the monoclonal antibody 4D1/C2 (bind to the linker between B and A2 domains) showed a 72 kDa in epithelial cells whereas in endothelial cells it was significantly higher with 95 kDa (Figure 31 right panel). Detection by using C5-1X revealed full length CEACAM1 in epithelial as well as in endothelial cells. In addition, a weak band of truncated CEACAM1 was detected in epithelial cell (Figure 31 middle panel). Further investigations were done to determine whether this 95 kDa variant mimic the N-domain truncated CEACAM1 detected in epithelial cells. Detection by using monoclonal antibody 18/20 did not detect the 95 kDa variant in endothelial cells (Figure 31 left panel). Therefore, indicating that the 95 kDa variant lacks the N-domain. However, epitope mapping using sandwich ELISA was controversial because it revealed this truncated CEACAM1 have N-domain. To elucidate this discrepancy, additional studies were done using deglycosylation assay. Subsequently, Western blot analysis using the monoclonal antibody 4D1/C2 revealed that deglycosylation of the truncated CEACAM1 reduced its molecular weight from 95 KDa to 40 kDa (Figure 32 right panel). This result clearly demonstrates that the 95 kDa CEACAM1 variant is glycosylated and represents not a deglycosylated variant of full length CEACAM1 in endothelial cells. This finding corresponds to the deglycosylated truncated CEACAM1 in epithelial cells (Figure 32 left panel). Although there was no 95 kDa CEACAM1 form detected in urine we suggest that the 95 kDa CEACAM1 variant released by endothelial cells is deglycosylated on the way to excretion to form a 72 kDa CEACAM1 form. Therefore, endothelial cells are probably an additional source of the 72 kDa truncated CEACAM1 variant found in urine.

5.4.1 Truncated CEACAM1 appears due to turnover processes in contact-inhibited endothelial cells

Next, we investigated whether this 95 kDa variant found in endothelial cells is upregulated similar to truncated CEACAM1 detected in epithelial cells. Western blot analysis using monoclonal antibodies 18/20 and C5-1X revealed a significant upregulation of full length

CEACAM1 when the cells were kept in contact inhibited state (Figure 33 left and Middle panel). Furthermore, Western blot analysis using monoclonal antibody 4D1/C2 revealed that the 95 kDa variant appeared in contact-inhibited (Figure 33 right panel). In addition, the 95 kDa CEACAM1 variant was significantly upregulated when the cells were kept in contact inhibited state. Further, we analyzed using sandwich ELSA the presence of CEACAM1 in the cell culture supernatants obtained from contact-inhibited endothelial cells. Our results revealed a continuous increases of released CEACAM1 (Figure 34A). To confirm these results immunoprecipitation was performed. Western blot analysis using 4D1/C2 revealed a weak band at 120 kDa and a broad band at 95 kDa in confluent plus four weeks samples (Figure 34 B left panel). However, detection using by the monoclonal antibody C5-1X revealed a weak band at 120 kDa, 95 kDa and a smear at 55 kDa. As shown in figure 34 no band was detected in the confluent samples. It was shown that CEACAM1 is expressed in endothelial cells of newly formed small vessels of angiogenic tissues such as in tumors, regeneration of endometrium, and placenta and wound healing, but not in quiescent blood vessels of normal human tissues (Ergun et al., 2000b). They also showed that once the blood vessels are stabilized CEACAM1 was not detected or weakly detected. Taken together, our results suggest a CEACAM1 turnover process in endothelial cells whereby the endothelial CEACAM1 expression is downregulated in form of truncated CEACAM1 once blood vessels switch from an angiogenic to a quiescent phenotype. However further studies are needed for the characterization of the 95 kDa variant found in endothelial cells.

5.5 Full length CEACAM1 is released from the cells in membrane-bound vesicles

It is well known that normal and malignant cells release part of their cell surface membrane to form circular membrane fragments called microvesicles (MV). Shedding of membrane-derived MV is physiological phenomenon that accompanies cell activation and growth (Beaudoin and Grondin, 1991; Tesse et al., 2006; Hugel et al., 2005). Interestingly, rapidly growing cell lines tend to secrete more MV than slowly growing cells. Generally, the number of MV shed from cells can be increased by factors such as cell activation, hypoxia, irradiation, oxidative injury, and shear stress (Hugel et al., 2005; Horstman et al., 2004; Barry and FitzGerald, 1999). MV contain numerals proteins and lipids similar to those present in the cell plasma (Hunter et al., 2008). Recently carcinoembryonic antigen (CEA), was observed on various MV shed from human breast carcinoma cells (Dolo et al., 1995).

We found that only truncated CEACAM1 is present in urine whereas epithelial and endothelial released both full length as well as truncated CEACAM1. To elucidate this discrepancy we analyzed the pellets obtained from urine after centrifugation. Immunobloting analysis revealed bands with 120 kDa which corresponds to the full length CEACAM1 but not to the truncated CEACAM1 variant (Figure 35). These data clearly demonstrates that full length CEACAM1 are released as membrane bound whereas truncated CEACAM1 are soluble because they were not detected in the urine pellet. This may imply that full length CEACAM1 found in the cell culture supernatants are also membrane bound and may have originated due to cell culture medium turning to acidic pH during cell culturing. However, further analyses on microvesicle containing CEACAM1 released by tumor cells are needed, furthermore, their biological functions.

5.6 Characterization of antibodies used in this study

The monoclonal antibodies used in this study were specifically binding to the various domains as follows: 18/20, R+D (N-domain), B3 (A1 domain), C5-1X (B domain) 4D1/C2 (the linker between B-A2 domains), 8G5 (A2 domain) and anti-CEACAM1 cyto (cytoplasmic region). They detected full length and truncated CEACAM1 as shown below (Table 17).

Immunoprecipitation was performed using only monoclonal antibodies that detected both full length and truncated CEACAM1. In addition, polyclonal antibody against CEA was used for immunoprecipitation because it binds to most CEACAM family members. Our results revealed that monoclonal antibody B3 precipitated full length CEACAM1 better than the truncated CEACAM1 whereas it was vice versa when monoclonal antibody 4D/1C2 was used. Also it was observed that monoclonal antibody C5-1X was not good for immunoprecipitation. Therefore it was not used for immunoprecipitation in this study.

Furthermore, we observed that in endothelial cell line AS-M.5 monoclonal antibody 4D1/C2 detected broad bands of truncated CEACAM1 and weak bands of full length CEACAM1 whereas, C5-1X detected broad bands of full length CEACAM1 and faint bands of truncated CEACAM1 although both monoclonal antibodies were not CEACAM1 N-domain binding. This was also observed in A549 epithelial cell. These differences may be due to the antibodies affinities or accessibility of their distinct epitopes domains. Therefore, our results clearly demonstrated that it is better to use C5-1X for detection of full length CEACAM1 and 4D1/C2 for detection of truncated CEACAM1 variant.

Interestingly, it was also noted that degylcosylation of CEACAM1 led to drastic reduction of the affinity by the detecting monoclonal antibody. This suggest that sugars are functional important for the binding of the antibody.

Monoclonal antibodies	Full length CEACAM1	Truncated CEACAM1
18/20	Yes	No
R+D	Yes	No
4D1/C2	Yes	Yes
C5-X1	Yes	Yes
B3	Yes	Yes
8G5	Yes	No
Anti-CEACAM1 (cyto)	Yes	No

Table 17 Binding of monoclonal antibodies to full length and truncated CEACAM1.

6. Summary

Previous studies have reported the presence of soluble CEACAM1 in serum, bile, saliva and seminal fluid, but its presence in urine was neglected (Draberova et al., 2000; Svenberg, 1976b). Nonetheless, CEACAM1 was detected most recently in urine of healthy donors and its concentration was even higher in patients suffering bladder cancer (Tilki et al., 2009). However, until now CEACAM1 in urine was not sufficiently characterized. Therefore, the analysis of the doctoral thesis presented here focused on the detailed characterization of CEACAM1 found in urine and the determination of its origin. Furthermore, we wanted to understand the cause leading to the CEACAM1 secretion and the mechanism behind.

CEACAM1 is a highly glycosylated cell adhesion receptor molecule. It is expressed on hematopoietic cells, most epithelia and angiogenicly activated endothelia. CEACAM1 plays an important role in cell morphogenesis, tumorigenesis, insulin metabolism, T-cell regulation and as a pathogen receptor. It is a member of the immunoglobulin superfamily and consists of one N-terminal V-like Ig domain, followed by two to three C2-like Ig domains [CEACAM1-4 (N-A1-B-A2); CEACAM1-3 (N-A1-B)]. In most cases the CEACAM1-4 splice variants are expressed. Depending on the glycosylation status of CEACAM1-4 the molecular weight ranges from 120 kDa to 160 kDa. Beside the transmembrane-bound form soluble full length CEACAM1 variants have been reported.

Our studies revealed that a high amount of CEACAM1 is present in the urine of healthy adults. Interestingly, with 72 kDa its molecular weight differed extensively compared to the known size of CEACAM1 at 120 kDa. To investigate the reason for this molecular weight reduction we established various sets of sandwich ELISA-systems, which enabled us to distinguish between the N-terminal domain, the A1B1 domain, the A2, domain and cytoplasmic domain, respectively. Our results showed that this novel CEACAM1 variant found in urine does not contain the N-domain, part of the A2 domain and cytoplasmic tail. Therefore, CEACAM1 in urine represents a so far unknown, truncated variant consisting of the A1, B and a part of the A2 domains. This finding was confirmed by Western blot analysis. Calculating the theoretical molecular weight of a CEACAM1 variant consiting of A1-B-part A2 very well agrees with the CEACAM1 version found in the urine.

As putative source of the truncated CEACAM1 we identified that cells expressing CEACAM1 endogenously (A549, T102/3, HT29,) and those transfected with CEACAM1 (Hela-CEACAM1) contained the native as well as the truncated form of CEACAM1. Interestingly, studies using cortex endothelial cell line (AS-M.5) revealed a CEACAM1 form with a significantly higher molecular weight of 95 kDa. However, domain mapping and deglycosylation studies revealed that the 95 kDa CEACAM1 variant found in endothelia concurred to the degylcosylated truncated CEACAM1 variant found in epithelial cells. Hence,

the molecular weight differences occurred just due to differences in the glycosylation level. Notably, no expression of truncated CEACAM1 was observed in granulocytes.

Further studies analyzing the generation of the truncated CEACAM1 variant showed that it appeared neither due to differences in deglycosylation nor its production induced by apoptosis. Furthermore, studies utilizing A549 epithelia cells, a cell type loosing its entire endogenous CEACAM1 expression by entering the proliferative stage, revealed that truncated CEACAM1 becomes not generated when full length CEACAM1 expression is down-regulated in epithelial cells entering cell proliferation.

Interestingly, our results showed for the first time that truncated CEACAM1 appeared in contact-inhibited cells. Extensive studies revealed that when cells were kept in the contact inhibited state a significant accumulation of truncated CEACAM1 appeared in the cell lysates and in the supernatant.

In summary, here we demonstrate for the first time a novel truncated form of CEACAM1 in human urine, which does not contain the N-domain, part of the A2 domain and cytoplasmic tail. The present data suggest that truncated CEACAM1 is generated during molecular turnover processes in epithelial and endothelial cells. Thus, this study leads to a better understanding of CEACAM1 turnover processes and the mechanism involved. Furthermore, this process might serve as a mechanism resulting in the production of functionally, an inactive CEACAM1 form since this truncated CEACAM1 lacks the ligand binding N-domain and the signal transducing cytoplasmic domain. Similar turnover processes and mechanism involved may be applicable to other adhesion receptors such as cadherins, selectins, and integrins.

7. Reference list

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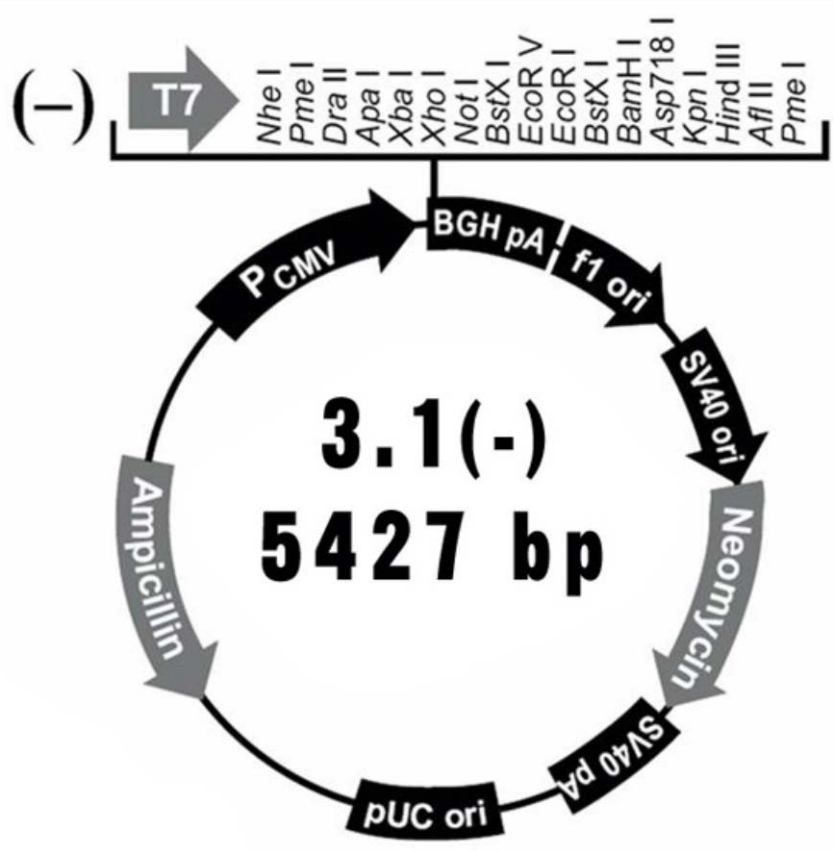
8. Supplementary data

8.1 Map of vector pcDNA 3.1 (-)



8.2 Publications

Harrison T. Muturi, Bernhard B. Singer Inka Scheffrahn, Derya Tilki, Süleyman Ergün. A novel soluble variant of human CEACAM1 appears due to molecular turnover processes in contact-inhibited differentiated epithelial and endothelial cells.
The manuscript is in preparation.

Posters and Presentations

1. **Harrison T. Muturi**, Inka Scheffrahn, Derya Tilki, Süleyman Ergün and Bernhard B. Singer. A novel truncated CEACAM1 form appears due to turnover processes in aging human epithelial and endothelial cells. 105th Annual Meeting of the Anatomische Gesellschaft, 26-29 March, 2010, Hamburg, Germany (Oral presentation).

2. **Harrison T. Muturi**, Inka Scheffrahn, Derya Tilki, Süleyman Ergün and Bernhard B. Singer. Identification and characterization of a human truncated CEACAM1. Annual Forschungstage, October 2009 University Hospital, Essen, Germany (Poster presentation).

3. **Harrison T. Muturi**, Inka Scheffrahn, Derya Tilki, Süleyman Ergün and Bernhard B. Singer. Identification and characterization of a novel truncated CEACAM1 form in human.19th Annual International CEA Symposium, 15-18 August,2009 Essen/Bochum-Wattenscheid, Germany (Poster & oral presentation).

4. **Harrison T. Muturi,** Inka Scheffrahn, Derya Tilki, Süleyman Ergün and Bernhard B. Singer. Identification and functional characterization of soluble CEACAM1 form. Annual Forschungstage, 30th October 2008 University Hospital, Essen, Germany (Poster presentation).

5. Bernhard B. Singer**, Harrison T. Muturi,** Inka Scheffrahn, Derya Tilki, and Süleyman Ergün. Identification and functional characterization of novel soluble forms of human CEACAM1. 18th Annual International CEA Syposium 7-10 September, 2008, Boston USA (Oral presentation).

6. Bernhard B. Singer**, Harrison T. Muturi,** Inka Scheffrahn, Derya Tilki, and Süleyman Ergün Identification and functional characterization of novel soluble forms of human CEACAM1. 103rd Annual Meeting of the Anatomische Gesellschaft 14-17 March, 2008, Innsbruck, Austria (Oral presentation).

8.3 Curriculum vitae

The biography is not included in the online version for reasons of data protection

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8.4 Declaration/Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„A novel soluble variant of human CEACAM1 appears due to molecular turn-over processes in contact-inhibited differentiated epithelial and endothelial cells."

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im Dezember 2010

Harrison Thuo Muturi

8.5 Acknowledgment

Along the Journey, I never walked alone. I have been supported and assisted by many people. I would like to express my heartfelt gratitude to Prof. Dr. med. Süleyman Ergün for giving me the opportunity to prove myself as a scientist at the Institute of Anatomy. I appreciate all his contributions, time, suggestions, helpful discussions, and funding in this work.

Special thanks to PD. Dr. Bernhard B. Singer for supervising this work skillfully and professionally. I appreciate all his insight comments, suggestions, and wide knowledge in science which has been of great value. I warmly thank him for teaching me many techniques in the lab and on how to thoroughly judge my results. Furthermore, he has been an understanding and encouraging friend who has stood by me in good and bad times.

I would like to express my thanks to Dr. Derya Tilki for assisting in funding my Ph.D studies and for been mindful of my financial status.

Thanks are also due to Dr. Inka Scheffrahn for her valuable ideas and recommendation during our fruitful discussions. I wish to warmly thank her for introducing and assisting me in the field of molecular biology.

In addition, I want to extend my deepest appreciation to Birthe Bongartz, Gudrun Mikus, Jens Moser, and Mohamed Salahdin Benchellal for their kind help and friendly assistance and other communication needs.

Thanks to Birgit Maranca-Hüwel and Jacky Klein for their sincere technical assistance and practical contribution to this work.

I wish also to recognize Dr. Veronika Kleff for her time, interest and assistance during the writing of this thesis.

My sincere thanks to all former and present members in Ergün´s group for their friendship and encouragement.

I also want to acknowledge wonderful friends whom I have walked with this journey so far. It’s virtually impossible to mention all of them by name.

Indeed this work would not have been possible without the enablement of my Lord and Savior Jesus Christ. His grace and strength has been overwhelming.

I have a large extended family and it’s impossible to recognize all those who have touched my life in a special way. But I do thank all of them for whatever role they played in my life. I do have some special people who have been at the centre of my life and who have filled it with hope and joy. First is my loving wife Emmah, for her moral support, and understanding. The path of my life did not make it easy for her. However, she has continued with steadfast love and encouragement. In a special way is my dear daughter Christabel and for the

generations that will follow. Finally, are my parents who embodied strength, wisdom, endurance, and faith in all my pursuits. Deepest appreciation to my parents-in-law.